CROWN ETHER DERIVATIVES

FIELD OF THE INVENTION

The invention relates to derivatives of crown ether chelators, including chromophoric and fluorescent derivatives, that are useful for chelating metal cations. Where the chelator is labeled with a fluorophore, it is an indicator useful for the detection, discrimination and quantification of metal cations. The chelators are optionally substituted one or more times with a chemically reactive group or a conjugated substance, such as a biological or nonbiological polymer, or a lipid.

BACKGROUND

Metal ions play an important role in biological systems. Cells utilize metal ions for a wide variety of functions, such as regulating enzyme activity, protein structure, cellular signaling, as catalysts, as templates for polymer formation and as regulatory elements for gene transcription. Metal ions can also have a deleterious effect when present in excess of bodily requirements or capacity to excrete. A large number of natural and synthetic materials are known to selectively or non-selectively bind to or chelate metal ions. Ion chelators are commonly used in solution for in vivo control of ionic concentrations and detoxification of excess metals, and as in vitro buffers. When bound to a fluorophore, ion chelators are typically used as optical indicators of ions and are useful in the analysis of cellular microenvironments or dynamic properties of proteins, membranes and nucleic acids.

Such indicators are also useful for measuring ions in extracellular spaces; in vesicles; in vascular tissue of plants and animals; biological fluids such as blood and urine; in fermentation media; in environmental samples such as water, soil, waste water and seawater; and in chemical reactors. Optical indicators for ions are important for qualitative and quantitative determination of ions, particularly in living cells. Fluorescent indicators for metal cations also permit the continuous or intermittent optical determination of these ions in living cells, and in solutions containing the ions.

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~~A variety of fluorescent indicators that are useful for the detection of biologically relevant soluble free metal ions (such as Ca^{2+}, Mg^{2+} and Zn^{2+}) have been described that utilize oxygen-containing anionic or polyanionic chelators to bind to metal ions. In particular, fluorescent indicators utilizing a polycarboxylate BAPTA chelator have been previously described (U.S. Patent No.: 4,603,209 to Tsien et al. (1986); U.S. Patent No. 5,049,673 to Tsien et al. (1991); U.S. Patent No. 4,849,362 to DeMarinis et al. (1989); U.S. Patent No. 5,453,517 to Kuhn et al. (1995); U.S. Patent No. 5,501,980 to Malekzadeh et al. (1996); U.S. Patent No. 5,459,276 to Kuhn et al. (1995); U.S. Patent No. 5,501,980 to Katerinopoulos et al. (1996); U.S. Patent No. 5,459,276 to Kuhn et al. (1995) (all incorporated by reference). Some fluorescent indicators selective for Li^+, Na^+ and K^+ in aqueous or organic solution have also been described, based on the chemical modification of crown ethers (U.S. Patent No. 5,134,232 to Tsien et al. (1992); U.S. Patent No. 5,405,975 to Kuhn et al. (1995); both incorporated by reference).~~

In general, a useful property for metal ion indicators is the ability to detect and/or quantify a selected metal ion in the presence of other metal ions. Discrimination of Ca^{2+}, Na^+ and K^+ ions in the presence of other metal ions is particularly useful for certain biological or environmental samples. For most biological applications, it is essential that the indicators be effective in aqueous solutions. It is also useful that indicators for biological applications be relatively insensitive to pH changes over the physiological range (pH 6-8) and sensitive to ion concentrations in the physiological range (for sodium, a K_d of about 5 mM to about 20 mM). It is also beneficial if the indicator absorbs and emits light in the visible spectrum where biological materials have low intrinsic absorbance or fluorescence.

Also useful are chelators that possess a chemically reactive functional group, so that the chelating group can be attached to polymers for use in remote sensing of ions or enhancing the solubility or localization of the optical sensor. Many chelators bind to intracellular proteins, altering the chelator's metal binding properties. In addition, due to their relatively small size, they are readily sequestered non-selectively in intracellular vesicles, further limiting their effectiveness. One means of circumventing these problems is to attach the desired crown

ether to a large, water-soluble polysaccharide, such as dextran or FICOL, by means of modification of the polysaccharide to allow covalent attachment of the indicator. Dextrans and FICOLs are especially suitable for this application, as they are low cost, optically transparent above about 250 nm and available in multiple ranges of molecular weights. Furthermore, polysaccharides and their conjugates are reasonably compatible with most biological materials and do not interact significantly with intracellular components. Although fluorescent polysaccharides have been previously described, as have indicator conjugates of dextrans, none possess the advantageous properties of the indicator conjugates of the current invention.

The crown ether chelators of the invention show significant ability to discriminate between metal ions under physiological conditions, particularly Ca^{2+}, Na^+ and K^+ ions. This selectivity can be tailored by careful selection of crown ether substituents. The compounds of the invention are typically soluble in aqueous solutions.

The compounds of the invention that act as indicators for target ions absorb and emit light in the visible spectrum and possess significant utility as a means of detecting and quantifying certain metal ion levels in living cells, biological fluids or aqueous solutions. Upon binding the target ion in the chelating moiety of the indicator, the optical properties of the attached fluorophore are generally affected in a detectable way, and this change is correlated with the presence of the ion according to a defined standard. Compounds having relatively long wavelength excitation and emission bands can be used with a variety of optical devices and require no specialized (quartz) optics, such as are required by indicators that are excited or that emit at shorter wavelengths. These indicators are suitable for use in fluorescence microscopy, flow cytometry, fluoroscopy, or any other application that currently utilize fluorescent metal ion indicators.

BRIEF DESCRIPTION OF THE DRAWINGS

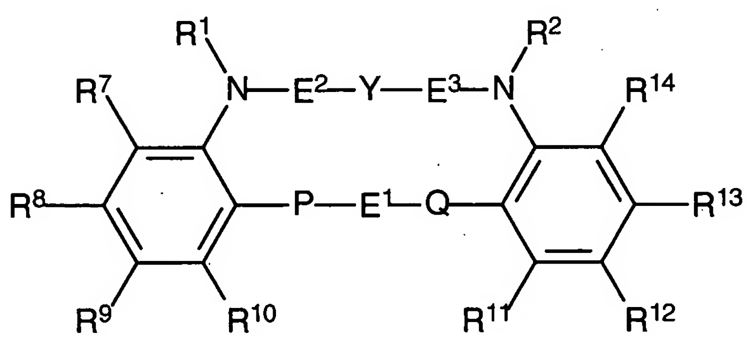
Fig 1. The Na^+-dependent fluorescence excitation spectra of Compound **167** in a series of solutions containing 0 to 1000 mM free Na^+, with fluorescence emission monitored at 510 nm (as described in Example 71).

Fig 2. The Na^+-dependent fluorescence emission spectra of Compound **132** in a series of solutions containing 0 to 500 mM free Na^+, with excitation at 488 nm (as described in Example 71).

Figure 3: A comparison of the intracellular sodium response of SODIUM GREEN tetraacetate indicator (Molecular Probes, Inc.) or Compound **134**. At every intracellular Na^+ concentration, Compound **134** demonstrates a stronger fluorescence intensity than SODIUM GREEN sodium indicator at the same concentration (as described in Example 72).

SUMMARY OF THE INVENTION AND DESCRIPTION OF THE PREFERRED EMBODIMENTS

The compounds of the invention have the formula



The heteroatoms P and Q are independently selected from O, S, or NR^3, where each R^3 is independently H or an alkyl having 1-6 carbons. In one aspect of the invention P and Q are both O.

The heteroatom Y is O, S, or NR^4, where R^4 is H, a C_1-C_{18} alkyl, or an aryl or heteroaryl ring system. The R^4 alkyl or ring system substituent is optionally substituted one or more times by halogen, azido, nitro, nitroso, amino, alkylamino having 1-6 carbons, dialkylamino having 2-12 carbons, cyano, or R^4 is substituted one or more times by a C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted one or more times by halogen,

amino, hydroxy, $-(SO_2)-R^{15}$, $-(SO_2)-O-R^{15}$, $-(C=O)-R^{15}$, $-(C=O)-O-R^{16}$, or $-(C=O)-NR^{17}R^{18}$. Alternatively, R^4 is, or is substituted by $-L-R_X$, $-L-S_C$, or -L-DYE.

Each R^{15} is independently H or C_1-C_6 alkyl. Each R^{16} is H, a C_1-C_6 alkyl, a benzyl, or forms an ester (e.g., R^{16} is an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or any other biologically compatible esterifying group). Additionally, any R^{16} is a biologically compatible salt. R^{17} and R^{18} are independently H or C_1-C_6 alkyl or C_1-C_6 carboxyalkyl, or an alpha-acyloxyalkyl, a t-butyldimethylsilyl, or any other biologically compatible esterifying group, or a biologically compatible salt; or R^{17} and R^{18} when taken in combination form a 5- or 6-membered aliphatic ring that optionally incorporates an oxygen atom. In addition, one or more of a R^{15}, R^{16}, R^{17}, or R^{18} is permitted to be $-L-R_X$, $-L-S_C$, or -L-DYE.

Each L is independently a covalent linkage. Each R_X is independently a reactive functional group. Each S_C is independently a conjugated substance. Each DYE is independently a moiety that maximally absorbs light at a wavelength greater than 320 nm.

In one aspect of the invention, P and Q are O, and Y is NR^4. In another aspect of the invention, P, Q, and Y are each O. Careful selection of the nature of the P, Q, and Y heteroatoms permits the moderation of the selectivity and binding affinity of the resulting crown ether.

E^1, E^2, and E^3 each independently have the formula $-(CR^5{}_2)_n-$, or $-[C(O)CH_2]_n-$, where n = 2-4. Each R^5 is independently H or methyl, or the R^5 moieties on adjacent carbon atoms of each chain, when taken in combination, form a 5- or 6-membered aliphatic ring. For a given E moiety, each R^5 is typically H and n is 2. Where n is 2 for each E moiety, the resulting compound is known as a 15-crown-5 crown ether, having 15 atoms in the chelating ring itself, of which 5 are heteroatoms.

The amine substituents R^1 and R^2 are independently H, C_1-C_{18} alkyl, or C_7-C_{18} arylalkyl. Where R^1 or R^2 is alkyl or arylalkyl, it is optionally substituted one or more times by halogen, azido, nitro, nitroso, amino, hydroxy, C_1-C_6 alkylamino, C_2-C_{12} dialkylamino,

cyano, or by an aryl or heteroaryl ring system , or by -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-$NR^{17}R^{18}$, where R^{15}, R^{16}, R^{17}, and R^{18} are as defined above; or by a C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted one or more times by halogen, amino, hydroxy, -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-$NR^{17}R^{18}$. Alternatively, R^1 and R^2 are optionally -L-R_X, -L-S_C, or -L-DYE.

For purposes of this invention, an aryl or aromatic ring system means a substituent containing 1 to 4 unsaturated rings (each ring containing 6 conjugated carbon atoms and no heteroatoms) that are optionally fused to each other or bonded to each other by carbon-carbon single bonds, and is optionally substituted as described below. Specific examples of an aryl ring system include, but are not limited to, substituted or unsubstituted derivatives of phenyl, biphenyl, *o*-, *m*-, or *p*-terphenyl, 1-naphthyl, 2-naphthyl, 1-, 2-, or 9-anthryl, 1-, 2-, 3-, 4-, or 9-phenanthrenyl and 1-, 2- or 4-pyrenyl. Preferably an aryl substituent is phenyl, substituted phenyl, naphthyl or substituted naphthyl.

For purposes of this invention, a heteroaryl or heteroaromatic ring system means a 5- or 6-membered unsaturated ring that is optionally fused to an additional six-membered aromatic ring(s), or is fused to one 5- or 6-membered unsaturated ring containing one or more heteroatoms, and is optionally substituted as defined below. Each heteroaromatic ring contains at least 1 and as many as 3 heteroatoms that are selected from the group consisting of O, N or S in any combination. Specific examples of a heteroaryl ring system include, but are not limited to, substituted or unsubstituted derivatives of 2- or 3-furanyl; 2- or 3-thienyl; N-, 2- or 3-pyrrolyl; 2- or 3-benzofuranyl; 2- or 3-benzothienyl; N-, 2- or 3-indolyl; 2-, 3- or 4-pyridyl; 2-, 3- or 4-quinolyl; 1-, 3-, or 4-isoquinolyl; 2-, 4-, or 5-(1,3-oxazolyl); 2-benzoxazolyl; 2-, 4-, or 5-(1,3-thiazolyl); 2-benzothiazolyl; 3-, 4-, or 5-isoxazolyl; N-, 2-, or 4-imidazolyl; N-, or 2-benzimidazolyl; 1- or 2-naphthofuranyl; 1- or 2-naphthothienyl; N-, 2- or 3-benzindolyl; 2-, 3-, or 4-benzoquinolyl; 1-, 2-, 3-, or 4-acridinyl. Preferably the heteroaryl substituent is substituted or unsubstituted 4-pyridyl, 2-thienyl, 2-pyrrolyl, 2-indolyl, 2-oxazolyl, 2-benzothiazolyl or 2-benzoxazolyl. More preferably, the heteroaryl substituent is 2-thienyl or 2-pyrrolyl.

Any aryl or heteroaryl ring system is unsubstituted or optionally and independently substituted by any synthetically accessible and chemically stable combination of substituents that are independently H, halogen, cyano, sulfo, alkali or ammonium salt of sulfo, nitro, carboxy, alkyl, perfluoroalkyl, alkoxy, alkylthio, amino, monoalkylamino, dialkylamino or alkylamido, typically the alkyl portions of which contain 18 or fewer carbons.

Where the R^1 and R^2 substituents are not -L-R_X, -L-S_C, or -L-DYE, they are typically both alkyl that is substituted one or more times by carboxylic acids, by carboxylic acid esters, by carboxylic acid amides, or by cyano. Where R^1 and R^2 incorporate carboxylic acid esters, they are optionally selected to be esters that are readily cleaved by intracellular esterase enzymes, such as alpha-acyloxyalkyl esters (typically acetoxymethyl esters). Alternatively, R^1 and R^2 incorporate carboxylic acid esters that are typically not cleaved, such as esters of lower alcohols (for example methyl or ethyl esters). In one embodiment, at least one of R^1, R^2, and R^7-R^{14} incorporates multiple carboxylic acids or carboxylic acid esters. Selection of the precise nature of R^1 and R^2 can greatly affect the selectivity and binding selectivity and affinity of the resulting compound (see Table 1).

The benzo substituents R^7-R^{14} are independently H, halogen, azido, nitro, nitroso, amino, cyano; or -L-R_X, -L-S_C, or -L-DYE; or C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted one or more times by halogen, amino, hydroxy, -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-N$R^{17}R^{18}$.

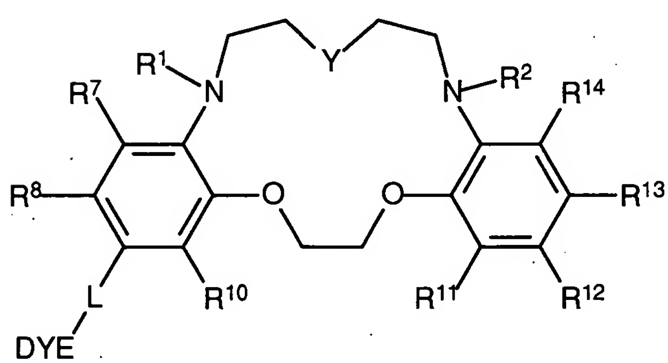
Alternatively, two adjacent substituents of R^7-R^{14}, when taken in combination, form a fused six-membered benzo moiety, which is optionally substituted one or more times by a halogen, azido, nitro, nitroso, amino, cyano, -L-R_X, -L-S_C, or -L-DYE; or by a C_1-C_6 alkyl or C_1-C_6 alkoxy, which is itself optionally substituted one or more times by halogen, amino, hydroxy, -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-N$R^{17}R^{18}$.

In one aspect of the invention, two adjacent substituents of R^7-R^{14}, when taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE.

The compounds of the invention are substituted by at least one -L-DYE, -L-R_X or -L-S_C at one or more of R^1, R^2, R^4, and R^7-R^{14}; or two of R^7-R^{14}, taken in combination, form a fused DYE. In one embodiment, the compound of the invention is substituted by exactly one -L-DYE moiety, which is bound at R^1, R^2, an R^4, or one of R^7-R^{14}, or is a fused DYE moiety at two adjacent substituents of R^7-R^{14}. The DYE moiety is typically bound at one of R^7-R^{11}, preferably at R^9, or is bound at R^4 where Y is NR^4. In one embodiment, compounds that are substituted by exactly one -L-DYE moiety are optionally further substituted by -L-R_x or -L--S_C, typically at R^1, R^2, R^4, or one of R^7-R^{14}.

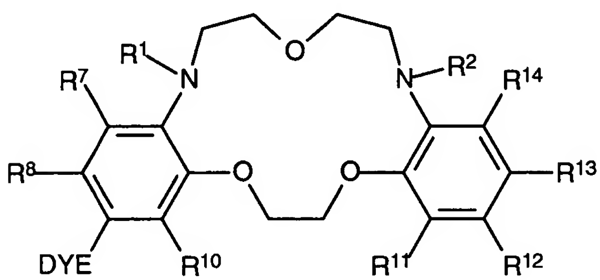
In another embodiment, the compound of the invention is substituted by exactly two DYE moieties, which may be the same or different, and may be bound by a covalent linkage L or fused to the crown ether chelate. In one embodiment of the invention, a first -L-DYE moiety is bound at one of R^7-R^{10}, while the second -L-DYE moiety is bound at one of R^{11}-R^{14}. Typically, the first -L-DYE moiety is bound at R^9, while the second -L-DYE moiety is bound at R^{12}, or a DYE moiety is fused at R^8 and R^9 and additionally at R^{12} and R^{13}.

In one aspect of the invention, the compound of the invention has the formula



where Y is either O or NR^4; and the DYE moiety is an indole, a coumarin, a stilbene, a xanthene, or a polyazaindacene. In this embodiment, preferably the DYE moiety is a xanthene, a polyazaindacene, or an oxazine.

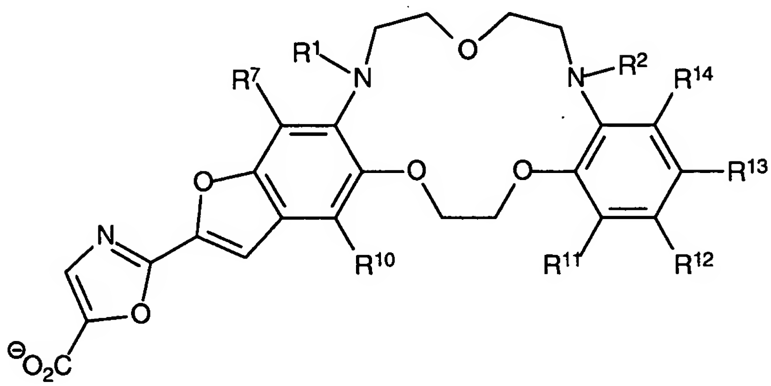
In one aspect of the invention, the compounds of the invention are fluorescent indicators having the following structure:



These indicators typically exhibit a low fluorescence quantum efficiency in the absence of metal ions. However, in the presence of increasing metal ion concentration the fluorescence quantum efficiency rises dramatically. For example, selected indicators of this family exhibit a fluorescence signal increase of over 100-times between zero and a saturating sodium concentration. Other selected indicators of the invention exhibit a shift of the wavelength of the absorption (excitation) maximum, emission maximum, or both, upon binding the target ion.

In one embodiment of this aspect, R^1 and R^2 are C_1-C_6 alkyl that are substituted one or more times by cyano, an aryl or heteroaryl ring system, or by -(C=O)-O-R^{16} or -(C=O)-$NR^{17}R^{18}$, where R^{16} is H, a C_1-C_6 alkyl, a benzyl, a biologically compatible esterifying group, or a biologically compatible salt; and R^{17} and R^{18} are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, an alpha-acyloxymethyl, or a biologically compatible salt. The substituents R^7 R^8, R^{10}, and R^{11}-R^{14}, are independently H, chloro, bromo, fluoro, nitro, amino, or cyano; or C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted by halogen, amino, hydroxy, -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-$NR^{17}R^{18}$. The DYE moiety is a polyazaindacene, an oxazine, or a xanthene, which is optionally substituted one or more times by halogen, nitro, sulfo, cyano, an aryl or heteroaryl ring system, or benzo, or alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, or carboxylic acids or carboxylic acid esters, the alkyl portions of which contain fewer than 20 carbons.

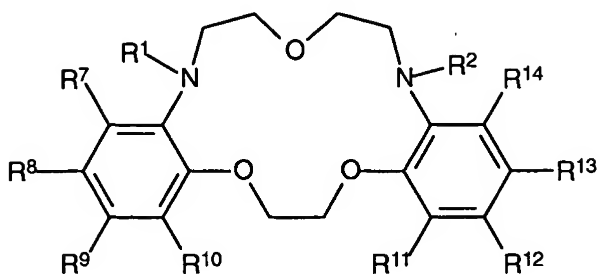
In another aspect of the invention, the compounds of the invention are fluorescent indicators having the following structure:



This class of indicators, in which at least one of the aromatic rings of the crown ether portion of the indicator is also incorporated in the DYE moiety, typically exhibit ratiometric fluorescence excitation changes in response to changing metal ion concentration. That is, there is a shift in the excitation maximum wavelength in the presence of increasing metal ion concentrations.

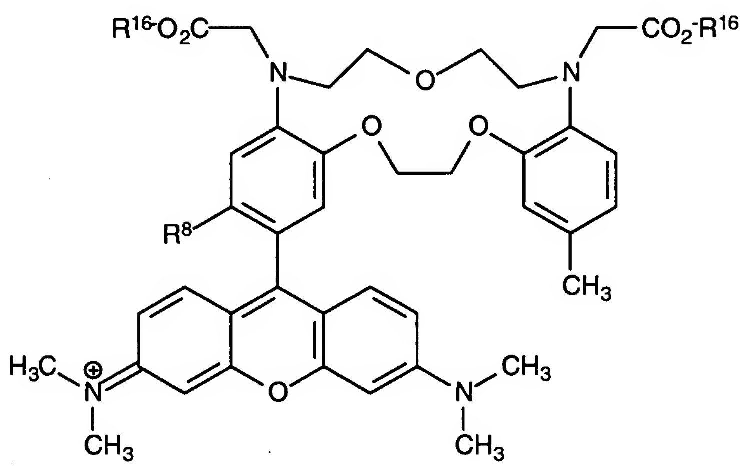
In one embodiment of this aspect, R^1 and R^2 are C_1-C_6 alkyl that are substituted one or more times by cyano, an aryl or heteroaryl ring system, or by -(C=O)-O-R^{16} or -(C=O)-$NR^{17}R^{18}$, where R^{16} is H, a C_1-C_6 alkyl, a benzyl, a biologically compatible esterifying group, or a biologically compatible salt; and R^{17} and R^{18} are independently H, C_1-C_6 alkyl, C_1-C_6 carboxyalkyl, an alpha-acyloxymethyl, or a biologically compatible salt. The substituents R^7, R^{10}, and R^{11}-R^{14}, are independently H, chloro, bromo, fluoro, nitro, amino, or cyano; or C_1-C_6 alkyl or C_1-C_6 alkoxy that is itself optionally substituted by halogen, amino, hydroxy, -(SO_2)-R^{15}, -(SO_2)-O-R^{15}, -(C=O)-R^{15}, -(C=O)-O-R^{16}, or -(C=O)-$NR^{17}R^{18}$.

The nature of the R^1 and R^2 substituents in large part determines the response of the indicators to particular target ions. For example, where the crown ether derivatives have the formula



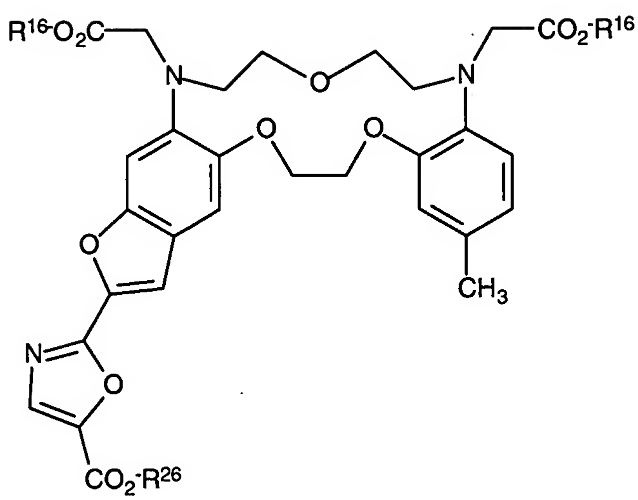
where R^1 and R^2 are each methoxycarbonylmethyl selectively typically bind sodium ions with a dissociation constant (K_d) of approximately 20-100 mM, and are relatively insensitive to the presence of potassium ions. In particular, the sodium ion K_d values typically rise less than about 10% when measured in the presence of 100 mM potassium ion.

An additional selected embodiment of the invention has the formula

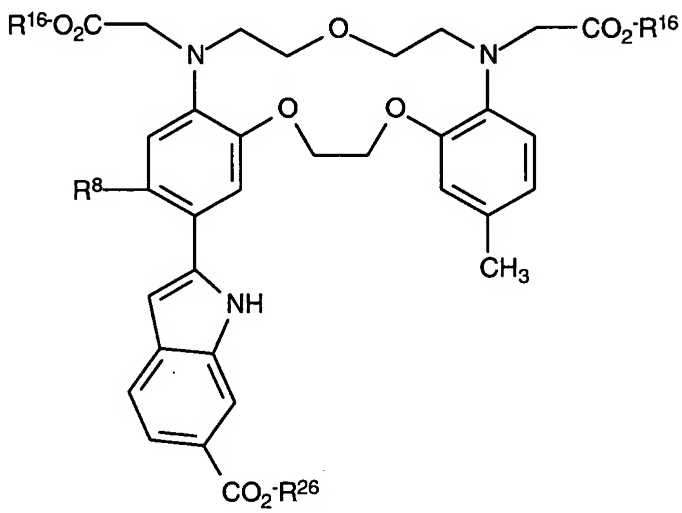


where R^8 and R^{16} are defined as above.

An additional selected embodiment of the invention has the formula

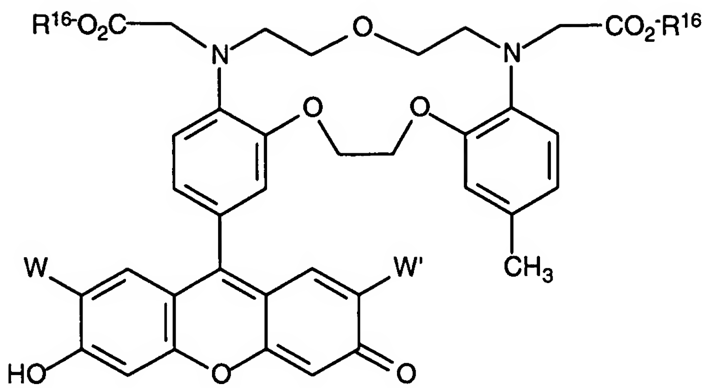


or the formula



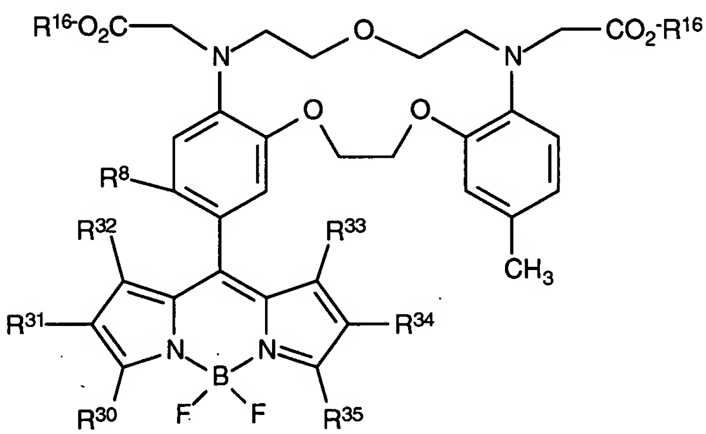
where R^8 and R^{16} are defined as above, and R^{26} is H, a C_1-C_6 alkyl, a benzyl, or is an alpha-acyloxyalkyl or a t-butyldimethylsilyl or other biologically compatible esterifying group, or is a biologically compatible salt.

An additional selected embodiment of the invention has the formula



where R^{16} is defined as above, and W and W' are independently F or Cl.

An additional selected embodiment of the invention has the formula



where R^8 and R^{16} are defined as above, and R^{30}-R^{35} are independently hydrogen, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, or acyl, wherein the alkyl portions of each contain fewer than 20 carbons; or aryl or heteroaryl ring system; or adjacent substituents R^{31} and R^{32}, and adjacent substituents R^{33} and R^{34}, when taken in combination form a fused benzo ring that is optionally substituted one or more times by hydrogen, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, alkylthio, alkylamido, amino, monoalkylamino or dialkylamino wherein the alkyl portions of each contain fewer than 20 carbons.

In an additional aspect of the invention, the compound of the invention further comprises a metal ion that is associated and/or complexes within the crown ether chelate portion of the compound. The metal ion is optionally a monocation (such as Li^+, Na^+, K^+, Rb^+, or Cs^+), a dication (such as Ca^{2+}, Zn^{2+}, or Mg^{2+}), or a polycation (such as Tb^{3+} or Eu^{3+}).

The DYE Moiety

The DYE moiety is any chemical moiety that exhibits an absorption maximum beyond 320 nm, that is bound to the crown ether chelate by a covalent linkage L, or that is fused to the crown ether chelate. The covalent linkage L is either a single covalent bond, or a combination of stable chemical bonds, as described in greater detail below. The covalent linkage binding the DYE moiety to the crown ether chelator is typically a single bond, but optionally incorporates 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S. In one embodiment, L is or incorporates a phenylene or a 2-carboxy-substituted phenylene.

Typically the DYE moiety contains one or more aromatic or heteroaromatic rings, that are optionally substituted one or more times by a variety of substituents, including without limitation, halogen, nitro, sulfo, cyano, alkyl, perfluoroalkyl, alkoxy, alkenyl, alkynyl, cycloalkyl, arylalkyl, acyl, aryl or heteroaryl ring system, benzo, or other substituents typically present on chromophores or fluorophores known in the art.

In one aspect of the invention, the DYE moiety has an absorption maximum beyond 480 nm. In a particularly useful embodiment, the DYE moiety absorbs at or near 488 nm to 514 nm (particularly suitable for excitation by the output of the argon-ion laser excitation source) or near 546 nm (particularly suitable for excitation by a mercury arc lamp). The DYE moiety may be a chromophore, resulting in a compound that acts as a chromogenic indicator, or more preferably, DYE is additionally a fluorophore, resulting in a compound that is a fluorescent indicator. Preferably, binding a target ion within the crown ether chelate results in a detectable optical response. As used herein, a detectable optical response means a change in, or occurrence of, an optical signal that is detectable either by observation or

instrumentally, such a change in absorption (excitation) wavelength, fluorescence emission wavelength, or fluorescence emission intensity.

Where the detectable response is a fluorescence response, it is typically a change in fluorescence, such as a change in the intensity, excitation or emission wavelength distribution of fluorescence, fluorescence lifetime, fluorescence polarization, or a combination thereof. Preferably, the detectable optical response upon binding the target metal ion is a change in fluorescence intensity that is greater than approximately 10-fold, more preferably greater than 50-fold. In another aspect, the detectable optical response upon binding the target metal ion is a shift in maximal excitation or emission wavelength that is greater than about 20 nm, more preferably greater than about 30 nm. Sodium and potassium indicators of the type that exhibit significant emission shifts have not been previously described.

A wide variety of chemically reactive fluorescent dyes that may be suitable for incorporation into the compounds of the invention are already known in the art (see for example MOLECULAR PROBES HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, Sixth Ed., Richard P. Haugland, ed. (1996), and Seventh Ed., Richard P. Haugland, ed. (1999) on CD-ROM; BIOPROBES 32 (December 1999); BIOPROBES 33 (February 2000); BIOPROBES 34 (May 2000); and BIOPROBES 35 (November 2000)). The spectral properties of candidate dyes in solution or when conjugated to proteins such as IgG are known or are readily measured using an absorption spectrometer or a spectrofluorometer.

In one aspect of the invention, the DYE moiety is a pyrene, an anthracene, a naphthalene, an acridine, a stilbene, an indole or benzindole, an oxazole or benzoxazole, a thiazole or benzothiazole, a 4-amino-7-nitrobenz-2-oxa-1,3-diazole (NBD), a cyanine, a carbocyanine, a carbostyryl, a porphyrin, a salicylate, an anthranilate, an azulene, a perylene, a pyridine, a quinoline, a coumarin (including hydroxycoumarins and aminocoumarins and fluorinated derivatives thereof (as described in U.S. Patent No. 5,830,912 (1998), incorporated by reference), a polyazaindacene (e.g., US Patents 4,774,339 to Haugland, et al. (1988); 5,187,288 to Kang, et al. (1993); 5,248,782 to Haugland, et al. (1993); 5,274,113 to

Kang, et al. (1993); and 5,433,896 to Kang, et al. (1995), all incorporated by reference), a xanthene, an oxazine or a benzoxazine, a carbazine (US Patent 4,810,636 to Corey (1989), incorporated by reference), or a phenalenone or benzphenalenone (US Patent 4,812,409 Babb et al. (1989), incorporated by reference). In another aspect of the invention, the DYE moiety is a carbazine, an oxazine, a coumarin, a pyrene, a xanthene, a naphthalene, a phenalenone, or a polyazaindacene. As used herein, oxazines include resorufins, aminooxazinones, diaminooxazines, and their benzo-substituted analogs.

Where the DYE moiety is a xanthene, the synthetic dye is optionally a fluorescein, a rhodol (US Patent 5,227,487 to Haugland, et al. (1993), incorporated by reference), or a rhodamine. As used herein, fluorescein includes benzo- or dibenzofluoresceins, seminaphthofluoresceins, or naphthofluoresceins. Similarly, as used herein rhodol includes seminaphthorhodafluors (U.S. Patent No. 4,945,171 to Haugland, et al. (1990), incorporated by reference). Fluorinated xanthene dyes have been described previously as possessing particularly useful fluorescence properties (Int. Publ. No. WO 97/39064, Molecular Probes, Inc. (1997), U.S. Patent No. 6,162,931 to Gee et al. (2000), incorporated by reference).

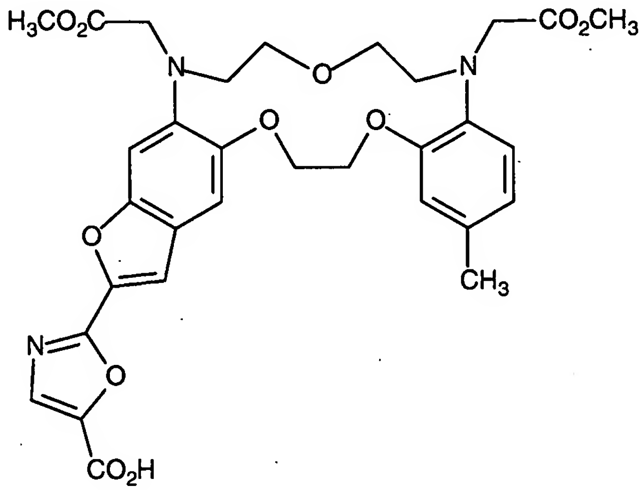
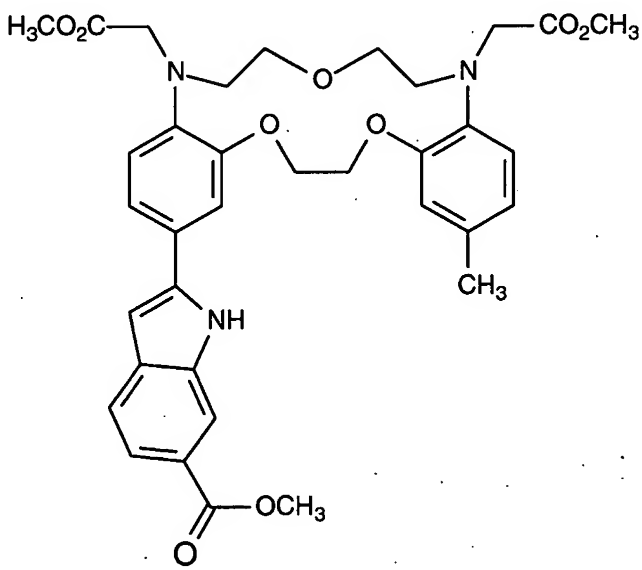
Alternatively, the DYE moiety is a xanthene that is bound via an L that is a single covalent bond at the 9-position of the xanthene. Preferred xanthenes include derivatives of 3*H*-xanthen-6-ol-3-one bound at the 9-position, derivatives of 6-amino-3*H*-xanthen-3-one bound at the 9-position, or derivatives of 6-amino-3*H*-xanthen-3-imine bound at the 9-position.

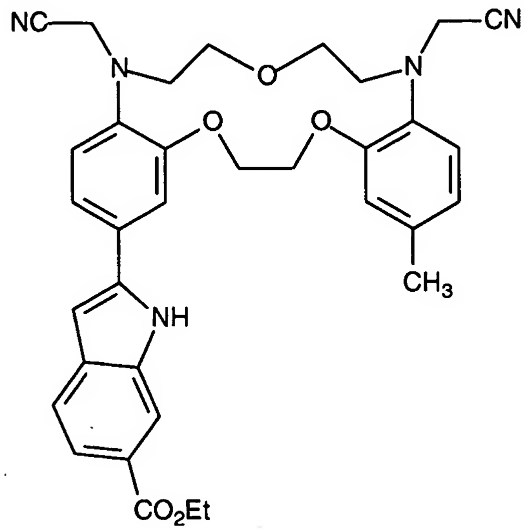
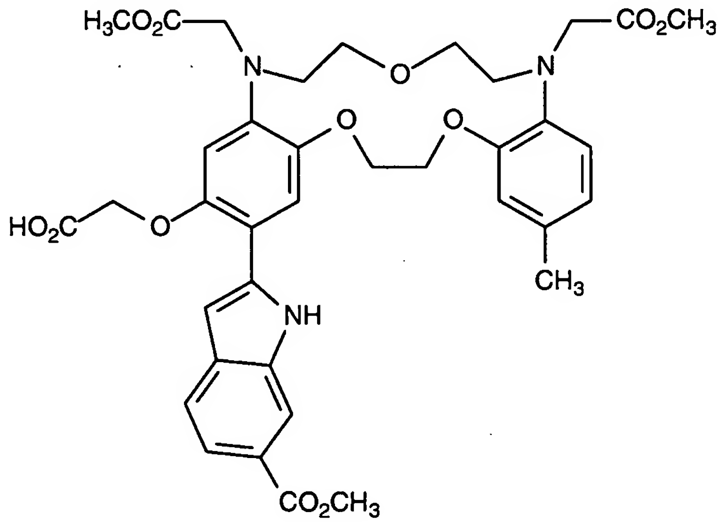
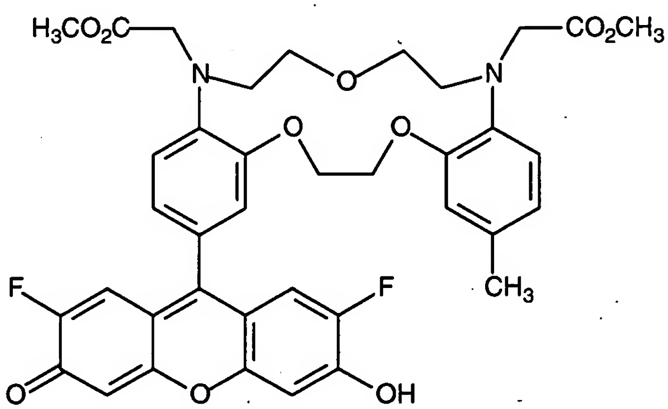
Selected sulfonated DYE moieties also exhibit advantageous properties, and include sulfonated pyrenes, coumarins, carbocyanines, and xanthenes (as described in U.S. Patent No. 5,132,432 to Haugland et al., (1992); U.S. Patent No. 5,696,157 to Wang et al. (1997); U.S. Patent No. 5,268,486 patent to Waggoner et al. (1993); U.S. Patent No. 6,130,101 to Mao et al. (2000); all incorporated by reference). Sulfonated pyrenes and coumarins are typically excited at wavelengths below about 450 nm.

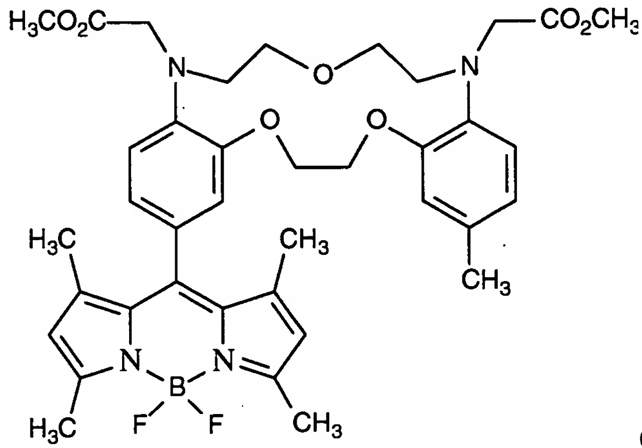
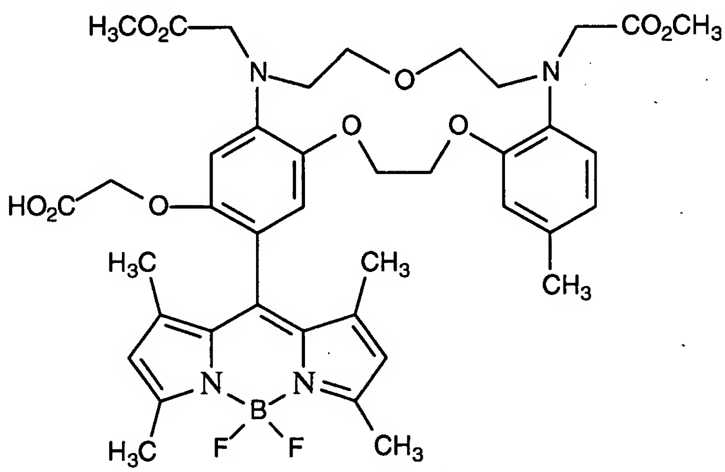
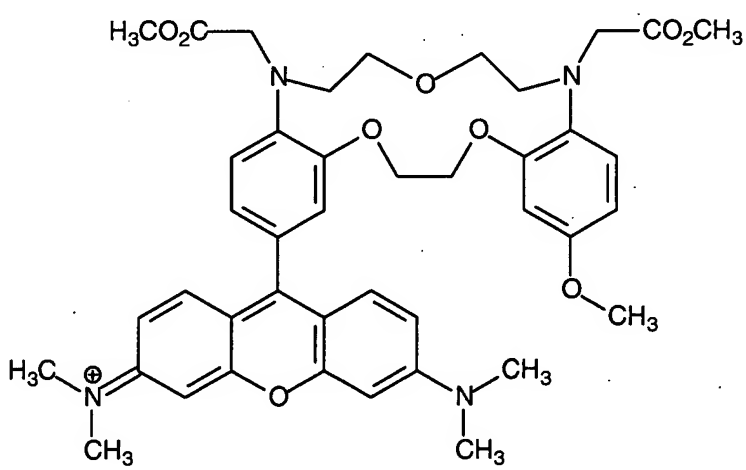
As described above, in one aspect of the invention, two adjacent substituents of R^7-R^{14}, when taken in combination with each other, and with the aromatic ring they are bound to, form a fused DYE. In one aspect of the invention, the fused DYE is a carbocyanine dye (as in Wang et al., J. WUHAN UNIV 2, 73 (1991)). In another embodiment, two adjacent ring substituents taken in combination form DYE moiety that is a fused benzofuran or heteroaryl- or carboxyheteroaryl-substituted benzofuran fluorophore, or a fused 6-membered unsaturated lactone, or benzazole-substituted lactone. Where the DYE moiety is fused to the compound of the invention, it is preferably fused R^8 and R^9, or at R^{12} and R^{13}.

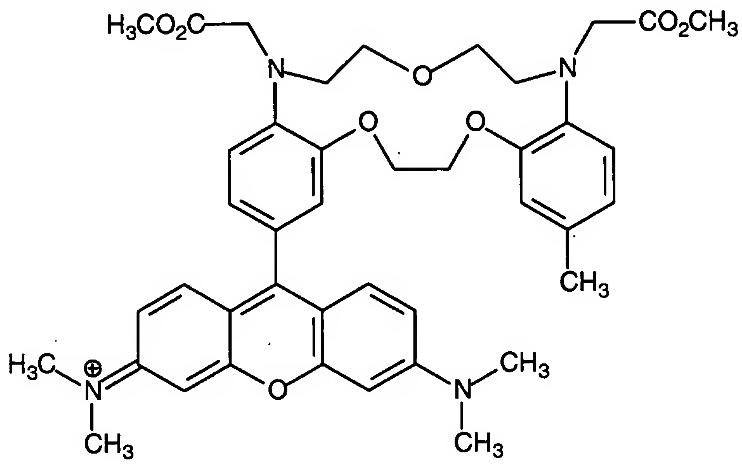
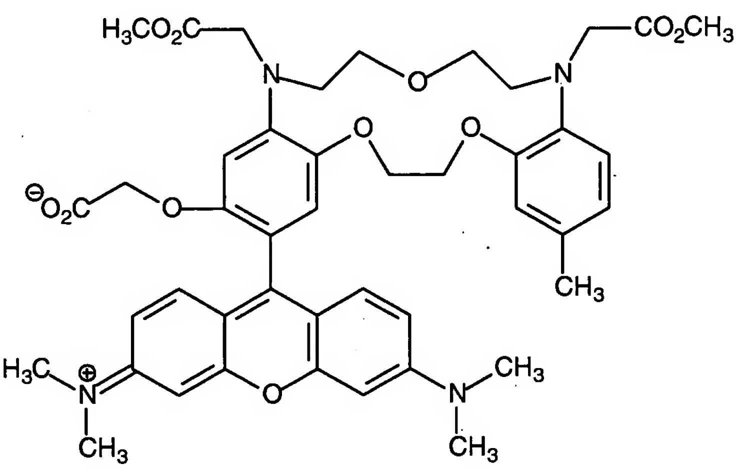
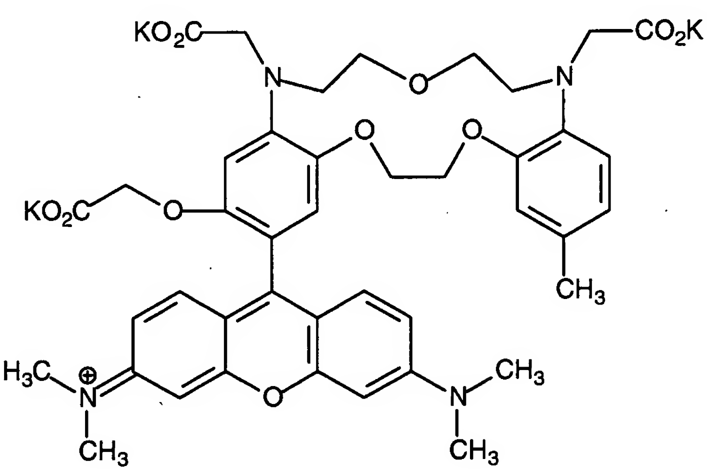
Selected embodiments of the invention are given in Table 1, showing a variety of distinct DYE moieties and crown ether substituents useful in the invention.

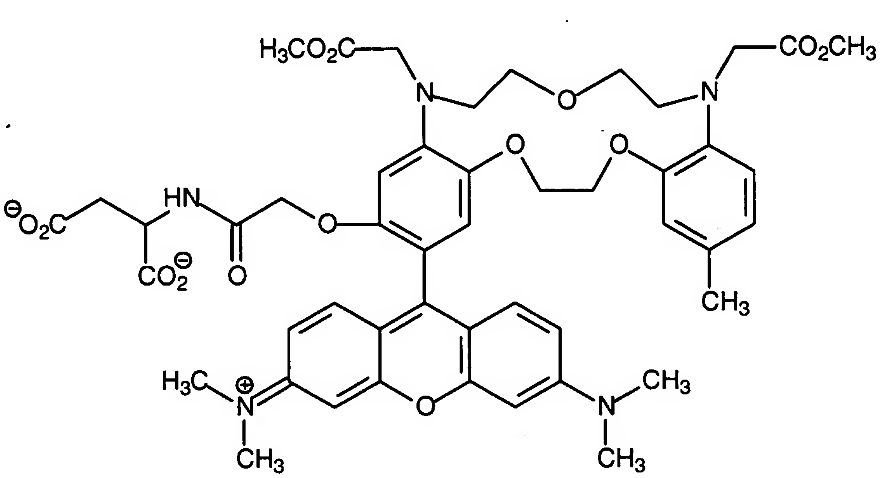
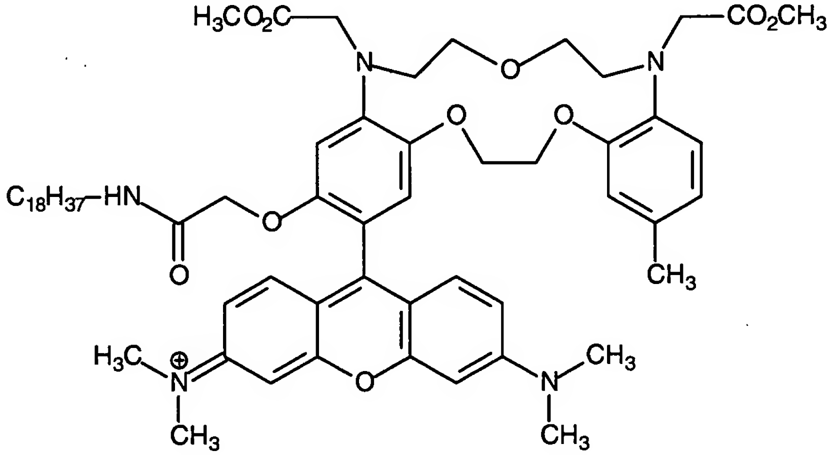
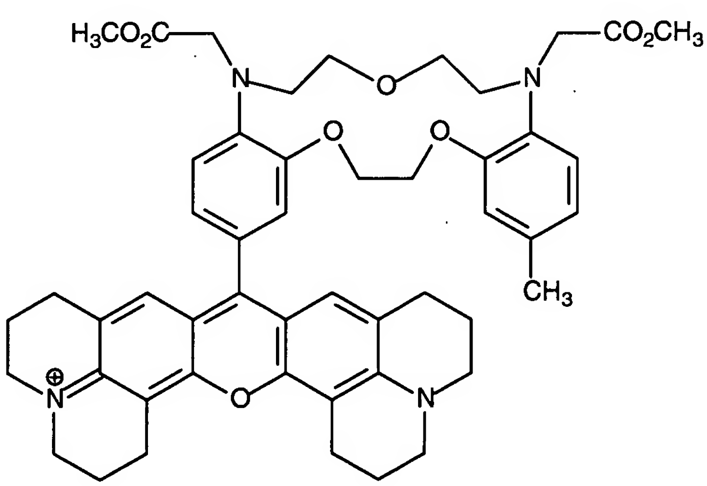
Table 1: Selected embodiments of the invention.

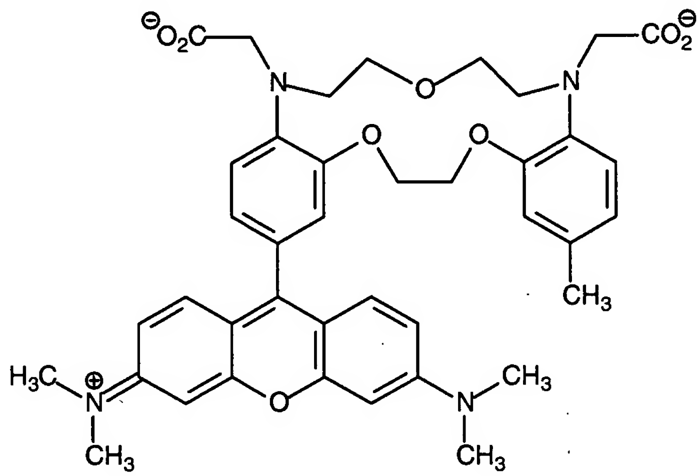
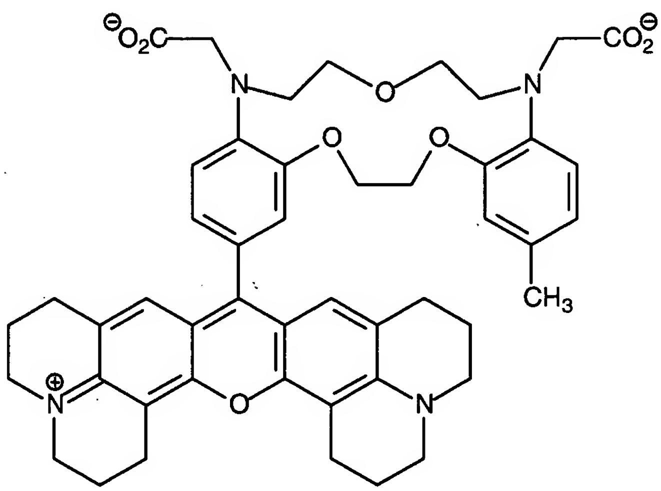
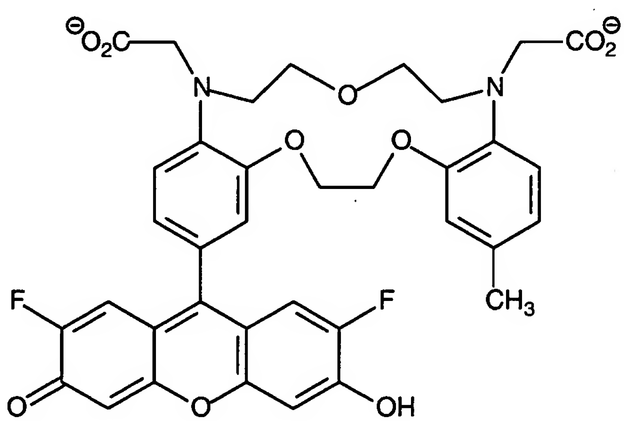
Compound	Dissociation Constant (target ion)
 Compound 167	K_d (Na^+) = ~ 52 mM K_d (K^+) = ~ 330 mM
 Compound 148	K_d (Na^+) = ~ 30 mM K_d (K^+) = ~ 115 mM

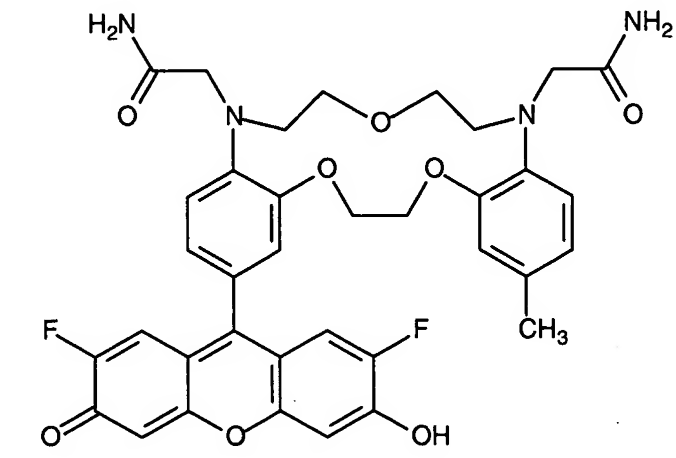
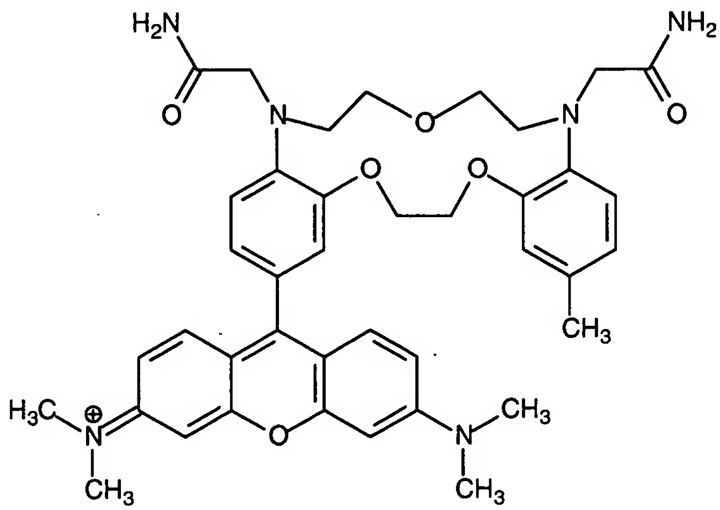
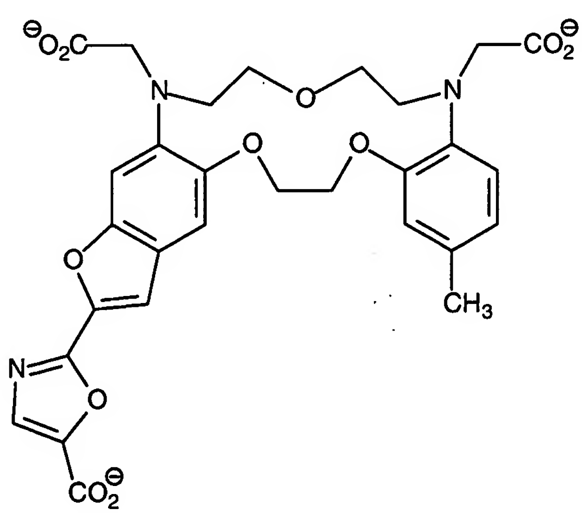
 Compound 180	K_d (Na^+) = ~220 mM
 Compound 174	K_d (Na^+) = ~ 52 mM K_d (K^+) = ~ 250 mM
 Compound 132	K_d (Na^+) = ~ 60 mM K_d (K^+) = ~ 205 mM

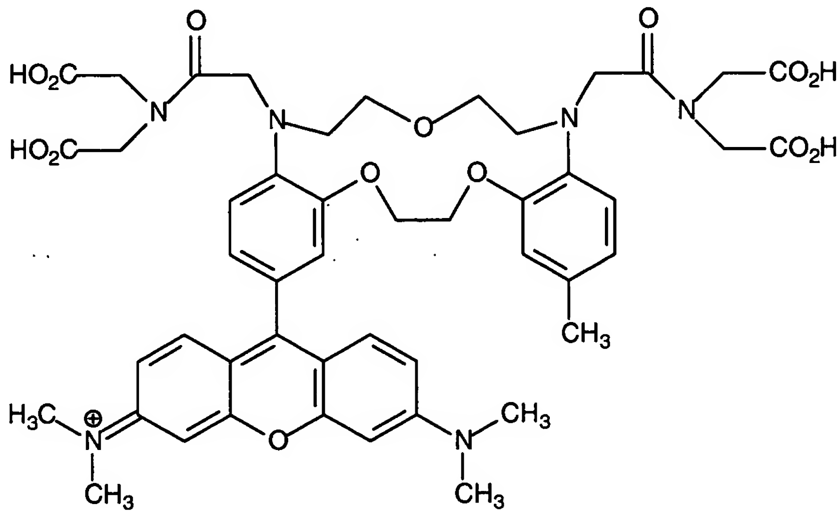
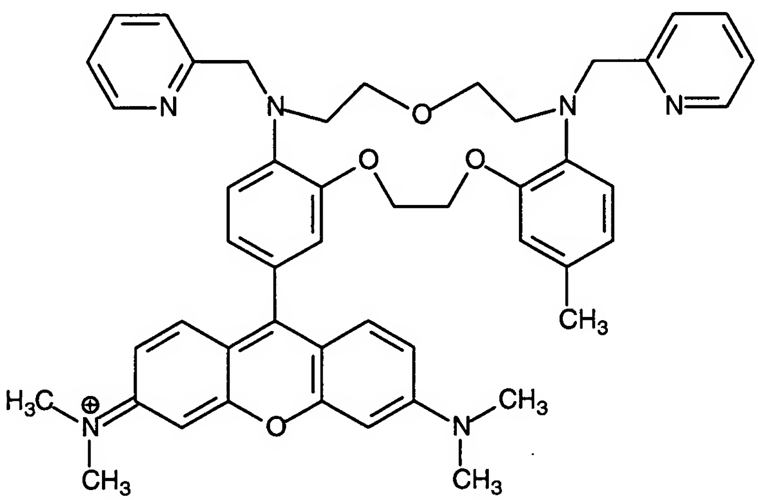
 Compound 145	K_d (Na^+) = ~ 42 mM
 Compound 164	K_d (Na^+) = ~ 28 mM K_d (K^+) = ~ 130 mM
	

 Compound 138	K_d (Na^+) = ~ 95 mM K_d (K^+) = ~ 300 mM
 Compound 152	K_d (Na^+) = ~ 92 mM K_d (K^+) = ~ 705 mM
 Compound 177	K_d (Na^+) = ~ 70 mM K_d (Zn^{2+}) = ~ 100 nM

	K_d (Na^+) = ~ 85 mM K_d (K^+) = ~ 255 mM
 Compound 156	K_d (Na^+) = ~ 14 mM
 Compound 143	K_d (Na^+) = ~ 103 mM K_d (K^+) = ~ 205 mM

 Compound 139	K_d (Zn^{2+}) = ~ 300 nM K_d (Ca^{2+}) = ~ 4 μM K_d (Na^{+}) = ~ 38 mM K_d (K^{+}) = ~ 270 mM
 Compound 144	K_d (Zn^{2+}) = ~ 8 μM K_d (Ca^{2+}) = ~ 7 μM
 Compound 133	K_d (Na^{+}) = ~ 36 mM K_d (K^{+}) = ~ 215 mM K_d (Zn^{2+}) = ~ 300 nM K_d (Ca^{2+}) = ~ 5 μM

	K_d (Na^+) = ~ 2.0 M K_d (Zn^{2+}) = ~ 600 μM
 Compound 182	K_d (Na^+) = ~ 500 mM K_d (Zn^{2+}) = ~ 650 μM
 Compound 171	K_d (Ca^{2+}) = ~ 400 nM K_d (Na^+) = ~ 160 mM

Reactive Functional Groups and Conjugated Substances

In one embodiment, the compound of the invention is substituted one or more times by an -L-R_X moiety, where R_X is the reactive group that is bound by an additional covalent linkage L, that may be the same or different from the covalent linkage that binds the DYE moiety to the crown ether chelator. In certain embodiments, the covalent linkage contains multiple intervening atoms that serve as a spacer. Compounds incorporating a reactive group (R_X) can be coupled to a wide variety of organic or inorganic substances that contain or are modified to contain functional groups with suitable reactivity, resulting in chemical attachment of the conjugated substance (S_C), represented by -L-S_C. The reactive group and functional group are typically an electrophile and a nucleophile that can generate a covalent linkage. Alternatively, the reactive group is a photoactivatable group, and becomes chemically reactive only after illumination with light of an appropriate wavelength. Typically, the conjugation reaction between the reactive group and the substance to be conjugated results in one or more atoms of the reactive group R_X to be incorporated into a new linkage L attaching the compound of the invention to the conjugated substance S_c. Selected examples of functional groups and linkages are shown in Table 2, where the reaction of an electrophilic group and a nucleophilic group yields a covalent linkage.

Table 2: Examples of some routes to useful covalent linkages

Electrophilic Group	Nucleophilic Group	Resulting Covalent Linkage
activated esters*	amines/anilines	carboxamides
acrylamides	thiols	thioethers
acyl azides**	amines/anilines	carboxamides
acyl halides	amines/anilines	carboxamides
acyl halides	alcohols/phenols	esters
acyl nitriles	alcohols/phenols	esters
acyl nitriles	amines/anilines	carboxamides
aldehydes	amines/anilines	imines
aldehydes or ketones	hydrazines	hydrazones

aldehydes or ketones	hydroxylamines	oximes
alkyl halides	amines/anilines	alkyl amines
alkyl halides	carboxylic acids	esters
alkyl halides	thiols	thioethers
alkyl halides	alcohols/phenols	ethers
alkyl sulfonates	thiols	thioethers
alkyl sulfonates	carboxylic acids	esters
alkyl sulfonates	alcohols/phenols	ethers
anhydrides	alcohols/phenols	esters
anhydrides	amines/anilines	carboxamides
aryl halides	thiols	thiophenols
aryl halides	amines	aryl amines
aziridines	thiols	thioethers
boronates	glycols	boronate esters
carbodiimides	carboxylic acids	N-acylureas or anhydrides
diazoalkanes	carboxylic acids	esters
epoxides	thiols	thioethers
haloacetamides	thiols	thioethers
haloplatinate	amino	platinum complex
haloplatinate	heterocycle	platinum complex
haloplatinate	thiol	platinum complex
halotriazines	amines/anilines	aminotriazines
halotriazines	alcohols/phenols	triazinyl ethers
halotriazines	thiols	triazinyl thioethers
imido esters	amines/anilines	amidines
isocyanates	amines/anilines	ureas
isocyanates	alcohols/phenols	urethanes
isothiocyanates	amines/anilines	thioureas
maleimides	thiols	thioethers
phosphoramidites	alcohols	phosphite esters

silyl halides	alcohols	silyl ethers
sulfonate esters	amines/anilines	alkyl amines
sulfonate esters	thiols	thioethers
sulfonate esters	carboxylic acids	esters
sulfonate esters	alcohols	ethers
sulfonyl halides	amines/anilines	sulfonamides
sulfonyl halides	phenols/alcohols	sulfonate esters

* Activated esters, as understood in the art, generally have the formula $-CO\Omega$, where Ω is a good leaving group (e.g., succinimidyloxy ($-OC_4H_4O_2$) sulfosuccinimidyloxy ($-OC_4H_3O_2-SO_3H$), -1-oxybenzotriazolyl ($-OC_6H_4N_3$); or an aryloxy group or aryloxy substituted one or more times by electron withdrawing substituents such as nitro, fluoro, chloro, cyano, or trifluoromethyl, or combinations thereof, used to form activated aryl esters; or a carboxylic acid activated by a carbodiimide to form an anhydride or mixed anhydride $-OCOR^a$ or $-OCNR^aNHR^b$, where R^a and R^b, which may be the same or different, are C_1-C_6 alkyl, C_1-C_6 perfluoroalkyl, or C_1-C_6 alkoxy; or cyclohexyl, 3-dimethylaminopropyl, or N-morpholinoethyl).

** Acyl azides can also rearrange to isocyanates

The covalent linkage L binds the reactive group R_X or conjugated substance S_C to the compound of the invention, either directly (L is a single bond) or with a combination of stable chemical bonds, optionally including single, double, triple or aromatic carbon-carbon bonds, as well as carbon-nitrogen bonds, nitrogen-nitrogen bonds, carbon-oxygen bonds, carbon-sulfur bonds, carbon-phosphorus bonds, phosphorus-oxygen bonds, phosphorus-nitrogen bonds, nitrogen-platinum bonds, and carbon-silicon bonds. L typically includes ether, thioether, carboxamide, sulfonamide, urea, urethane or hydrazine moieties. In one embodiment, the covalent linkage incorporates a platinum atom, such as described in U.S. Patent No. 5,714,327 (incorporated by reference).

Preferred L moieties have 1-20 nonhydrogen atoms selected from the group consisting of C, N, O, P, and S; and are composed of any combination of ether, thioether, amine, ester, carboxamide, sulfonamide, hydrazide bonds and aromatic or heteroaromatic bonds. Preferably L is a combination of single carbon-carbon bonds and carboxamide or thioether bonds. The longest linear segment of the linkage L preferably contains 4–10 nonhydrogen

atoms, including one or two heteroatoms. Examples of L include substituted or unsubstituted polymethylene, arylene, alkylarylene, arylenealkyl, or arylthio.

In one embodiment, L contains 1-6 carbon atoms; in another, L comprises a thioether linkage. In another embodiment, L is or incorporates the formula $-(CH_2)_d(CONH(CH_2)_e)_z-$ or $-O(CH_2)_d(CONH(CH_2)_e)_z-$, where d is an integer from 0-5, e is an integer from 1-5 and z is 0 or 1. In a further embodiment, L is or incorporates the formula $-O-(CH_2)-$. In yet another embodiment, L is or incorporates a phenylene or a 2-carboxy-substituted phenylene.

Choice of the reactive group used to attach the compound of the invention to the substance to be conjugated typically depends on the functional group on the substance to be conjugated and the type or length of covalent linkage desired. The types of functional groups typically present on the organic or inorganic substances include, but are not limited to, amines, amides, thiols, alcohols, phenols, aldehydes, ketones, phosphates, imidazoles, hydrazines, hydroxylamines, disubstituted amines, halides, epoxides, silyl halides, carboxylate esters, sulfonate esters, purines, pyrimidines, carboxylic acids, olefinic bonds, or a combination of these groups. A single type of reactive site may be available on the substance (typical for polysaccharides or silica), or a variety of sites may occur (e.g., amines, thiols, alcohols, phenols), as is typical for proteins. A conjugated substance may be conjugated to more than one dye, which may be the same or different, or to a substance that is additionally modified by a hapten, such as biotin. Although some selectivity can be obtained by careful control of the reaction conditions, selectivity of labeling is best obtained by selection of an appropriate reactive functional group.

Typically, R_X will react with an amine, an alcohol, an aldehyde, a ketone, or with silica. Preferably R_X reacts with an amine or a thiol functional group, or with silica. In one embodiment, R_X is an acrylamide, an activated ester of a carboxylic acid, an acyl azide, an acyl nitrile, an aldehyde, an alkyl halide, a silyl halide, an anhydride, an aniline, an aryl halide, an azide, an aziridine, a boronate, a diazoalkane, a haloacetamide, a halotriazine, a hydrazine (including hydrazides), an imido ester, an isocyanate, an isothiocyanate, a maleimide, a phosphoramidite, a reactive platinum complex, a sulfonyl halide, or a thiol

group. By "reactive platinum complex" is particularly meant chemically reactive platinum complexes such as described in U.S. Patent No. 5,714,327 (incorporated by reference).

Where R_X is an activated ester of a carboxylic acid, the resulting compound is particularly useful for preparing conjugates of proteins, nucleotides, oligonucleotides, or haptens. Where R_X is a maleimide or haloacetamide the resulting compound is particularly useful for conjugation to thiol-containing substances. Where R_X is a hydrazide, the resulting compound is particularly useful for conjugation to periodate-oxidized carbohydrates and glycoproteins, and in addition is an aldehyde-fixable polar tracer for cell microinjection. Where R_X is a silyl halide, the resulting compound is particularly useful for conjugation to silica surfaces, particularly where the silica surface is incorporated into a fiber optic probe subsequently used for remote ion detection or quantitation.

Preferably, R_X is a succinimidyl ester of a carboxylic acid, a haloacetamide, a hydrazine, an isothiocyanate, a maleimide group, an aliphatic amine, a silyl halide, or a psoralen. More preferably, R_X is a succinimidyl ester of a carboxylic acid, a maleimide, an iodoacetamide, or a silyl halide. In a particular embodiment R_X is a silyl halide or an isothiocyanate.

The compounds of the invention that possess a reactive functional group are useful for conjugation to any substance that possesses a suitable functional group for covalent attachment of the chelate. Examples of particularly useful conjugates include, among others, conjugates of antigens, steroids, vitamins, drugs, haptens, metabolites, toxins, environmental pollutants, amino acids, peptides, proteins, nucleic acids, nucleic acid polymers, carbohydrates, lipids, and non-biological polymers. Alternatively, these are conjugates of cells, cellular systems, cellular fragments, or subcellular particles. Examples include, among others, virus particles, bacterial particles, virus components, biological cells (such as animal cells, plant cells, bacteria, yeast, or protists), or cellular components.

Preferably the conjugated substance is a protein, polysaccharide, lipid, lipid assembly, non-biological polymer, or polymeric microparticle. Another class of preferred conjugated

substances includes particles or fibers composed of silica or other glasses, useful for preparing optical devices for remote sensing.

Preferred nucleic acid polymer conjugates are labeled, single- or multi-stranded, natural or synthetic DNA or RNA, DNA or RNA oligonucleotides, or DNA/RNA hybrids, or incorporate an unusual linker such as morpholine derivatized phosphates (AntiVirals, Inc., Corvallis OR), or peptide nucleic acids such as *N*-(2-aminoethyl)glycine units.

In a preferred embodiment, the conjugated substance (S_c) is a carbohydrate that is typically a polysaccharide, such as a dextran, FICOLL, heparin, glycogen, amylopectin, mannan, inulin, starch, agarose and cellulose. Alternatively, the carbohydrate is a polysaccharide that is a lipopolysaccharide. Preferred polysaccharide conjugates are dextran, FICOLL, or lipopolysaccharide conjugates.

In another embodiment, the conjugated substance (S_c), is a lipid moiety (typically having 6–60 carbons), including glycolipids, phospholipids, sphingolipids, and steroids. Alternatively, the conjugated substance is a lipid assembly, such as a liposome. The lipid moiety may be used to retain the conjugated substances in cells, as described in U.S. Pat. 5,208,148 (incorporated by reference).

Other conjugates of non-biological materials include conjugates of organic or inorganic polymers, polymeric films, polymeric wafers, polymeric membranes, polymeric particles, or polymeric microparticles, including magnetic and non-magnetic microspheres, conducting and non-conducting metals and non-metals, and glass and plastic surfaces and particles. Conjugates are optionally prepared by copolymerization of a compound of the invention that contains an appropriate functionality while preparing the polymer, or by chemical modification of a polymer that contains functional groups with suitable chemical reactivity. Other types of reactions that are useful for preparing conjugates of polymers include catalyzed polymerizations or copolymerizations of alkenes and reactions of dienes with dienophiles, transesterifications or transaminations. In another embodiment, the conjugated substance is a glass or silica, which may be formed into an optical fiber or other

structure.

Conjugates typically result from mixing appropriate reactive dyes and the substance to be conjugated in a suitable solvent in which both are soluble. Labeling of insoluble polymers or silica can be performed in a suspension of the insoluble polymer in a suitable solvent. For those reactive groups that are photoactivated, conjugation requires illumination of the reaction mixture to activate the reactive group.

Synthesis

There are typically three components to the methodology used to prepare the compounds of the invention. The first involves the formation of the crown ether chelate itself, the second involves the appropriate derivatization of the secondary amine nitrogen atoms of the crown ether chelate, and the third involves modification of the crown ether chelate by forming a reactive functional group, covalently attaching a conjugate, or covalently attaching a DYE moiety to form an indicator. Although these synthetic components are typically performed in the order given, they may be carried out in any other suitable sequence. For example, a portion of the chelate may be derivatized with a fluorescent dye prior to formation of the complete chelate ring.

Where the P and Q moieties are both oxygen, and E^1 is ethylene, the crown ether chelate is typically prepared by acylation of a bis-(2-aminophenoxy)ethane with a bis-(acid chloride), such as diglycolyl chloride, followed by reduction of the resulting bis-amide to the corresponding bis-secondary amine. Selection of the appropriate bis-acid chloride results in the particular desired crown ether (as in Examples 64-69 and 70).

The secondary amine nitrogen atoms present in the crown ether are typically derivatized with an alkylating agent. As the metal binding ability of the resulting crown ether is significantly influenced by the nature of the amine substituents, careful selection of the alkylating agent may be necessary to prepare a reporter for a particular target ion. Where the crown nitrogens are alkylated by methyl bromoacetate, the resulting bis-aza-crown ether is

typically selective for sodium ions. If the alkylating agent is 2-picolyl chloride, the resulting crown ether is typically selective for zinc ions. As discussed above, the presence of esters vs. carboxylic acids on the amine nitrogen substituents may influence the relative binding affinity of selected target ions. Selection of an alkylating agent that incorporates a precursor to a reactive functional group is useful for producing chemically reactive compounds of the invention, as well as acting as a useful intermediate for preparing conjugates, as described above. Additionally, an alkylating agent that incorporates a reporter DYE results in a crown ether compound that functions as an indicator for selected target ions.

More typically, the crown ether chelate is derivatized at the benzo ring of the crown ether. As described above, typically a suitable crown ether is prepared, and then bound to a DYE moiety. In one aspect of the invention, an ortho-hydroxy aromatic aldehyde is treated with a chloromethyl heterocycle to yield a fused reporter (as described in Example 46). In another aspect of the invention, derivatization with a DYE moiety is carried out by modifying a crown ether that possesses an aldehyde or ketone functional group.

In one aspect of the invention, the crown ether is substituted by an aldehyde and the fluorophore precursors and the crown ether are combined under anaerobic or non-oxidative conditions (e.g., under nitrogen), and subsequently oxidized using a mild oxidant (e.g., a quinone oxidant, preferably DDQ or chloranil). Where xanthene fluorophore precursors are condensed under anaerobic conditions, the resulting fluorophore is the non-fluorescent dihydro species, which may be utilized without prior oxidation as a sensor for oxidative subenvironments, e.g., in cells.

In yet another aspect of the invention, the crown ether is substituted by a carboxylic acid or by an aldehyde that is converted to the carboxylic acid in the course of synthesis of the crown ether. The chelating moiety is then condensed with the fluorophore precursors to yield the resulting indicator directly.

Synthesis of conventional xanthene dyes such as fluoresceins, rhodamines and rhodols typically involve the condensation of two equivalents of resorcinol (for fluoresceins),

aminophenol (for rhodamines) or a mixture of a resorcinol and an aminophenol (for rhodols) with a carbonyl-containing moiety such as a phthalic acid derivative or benzaldehyde. In the synthesis of the xanthene indicators of the invention, the desired resorcinol or aminophenol is condensed with the substituted crown ether, yielding either the reduced xanthene (where the crown ether contains an aldehyde) or the oxidized xanthene (where the crown ether contains a carboxylic acid or acyl halide) bound directly to the chelating moiety.

An oxidation step is typically required after condensation of a formyl-substituted crown ether with the fluorophore precursors. Optionally, the dihydro condensation product is isolated and subsequently oxidized with air or by standard chemical oxidants, such as chloranil. For some fluorophores, the oxidation reaction is enhanced by acidic reaction conditions. These mild oxidation reaction conditions tolerate a wide variety of substituents on the fluorophore and/or crown ether of the resulting indicators.

Unsymmetrical xanthene dyes are typically constructed by statistical methods, using a 1:1 mixture of the desired resorcinols or aminophenols in the condensation reaction, and purifying the desired product from the statistical mix of products using methods known in the art.

The synthesis of polyazaindacene dyes, particularly dipyrrometheneboron difluoride dyes, has been well documented (U.S. Patent No. 4,774,339, U.S. Patent No. 5,187,288, U.S. Patent No. 5,248,782, U.S. Patent No. 5,274,113, U.S. Patent No. 5,338,854, and U.S. Patent No. 5,433,896, all incorporated by reference). The procedure typically consists of an acid-catalyzed condensation of a benzaldehyde with a pyrrole that has a hydrogen at the 2-position, followed by *in situ* oxidation of the condensed intermediate by air, oxygen or a chemical oxidant such as 2,3-dichloro-5,6-dicyano-1,4-benzoquinone. The condensation of two appropriately substituted pyrroles, each having a hydrogen at the 2-position, with a formyl-substituted crown ether, followed by *in situ* oxidation of the condensed intermediate and treatment with a boron trifluoride etherate yields the dipyrrometheneboron difluoride indicators of the invention. Alternatively, the indicators are formed via the direct condensation of a carboxyl- or chlorocarbonyl-substituted crown ether with two equivalents

of appropriately substituted pyrroles, which may be the same of different, provided each has a hydrogen at the 2-position. The latter procedure does not require oxidation.

Post-condensation modifications of both the crown ether and the fluorophore moiety are typically strictly analogous to known methods of indicator modification. For example, the reduction of nitro substituents to amino groups, the conversion of carboxy substituents to cyano groups, and the preparation of esters of carboxylic acids, including acetoxymethyl esters. Additionally, salts and counterions of the indicators of the invention are readily converted to other salts by treatment with ion-exchange resins, selective precipitation, and basification, as is well-known in the art.

Post-condensation modifications of xanthylium dyes are well known. For instance, the xanthenone portion of the dye can be halogenated by treatment with the appropriate halogenating agent, such as liquid bromine. Xanthenes containing unsaturated fused rings can be hydrogenated to the saturated derivatives.

The reduced and oxidized versions of the xanthene indicators are freely interconvertible by well-known oxidation or reduction reagents, including borohydrides, aluminum hydrides, hydrogen/catalyst, and dithionites. Care must be exercised to select an oxidation or reducing agent that is compatible with the crown ether chelator. A variety of oxidizing agents mediate the oxidation of dihydroxanthenes, including molecular oxygen in the presence or absence of a catalyst, nitric oxide, peroxynitrite, dichromate, triphenylcarbenium and chloranil. The dihydroxanthenes are also oxidized electrochemically, or by enzyme action, including horseradish peroxidase in combination with peroxides or by nitric oxide.

Rather than condensing the DYE moiety precursors directly with a substituted crown ether, the preformed DYE moiety may be covalently bound to the crown ether via a conventional cross-linking reaction. A wide variety of chemically reactive or potentially chemically reactive and fluorescent fluorescein, rhodamine, rhodol, benzoxanthenes, dibenzoxanthene and other xanthene oxygen heterocycles that absorb maximally beyond

about 490 nm are commercially available as described by Haugland, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS (7th ed., 1999), as described above, or in other literature references. The nature of the bond that links DYE moiety to the crown ether chelate appears to have an effect on the optical response of the DYE moiety to ion binding, sometimes a significant effect. Acceptability of the linking chemistry can be determined by titration of the resultant indicator with the ion of interest over the target range of response (as described in Example 71).

METHOD OF USE

The crown ether compounds of the invention are useful for any application where it is desirable to complex a target metal ion. Selected crown ether compounds of the invention may be useful as ionophores, that is, they facilitate the transport of selected target ions across cell membranes. Where the crown ether compound is bound to a conjugated substance that is a polymeric matrix, such as a microparticle, or agarose, the compounds are useful for depleting a sample solution of a selected target ion, particularly where the polymeric matrix is used to pack a chromatography column. Other crown ether compounds (those bound to a DYE moiety) are useful as colorimetric or fluorescent indicators for a selected target ion.

In order for a particular indicator of the present invention to be useful, it must exhibit a detectable change in spectral properties upon complexation of the desired metal ion (target ion) in the chelating moiety. Preferably the change in spectral properties is a change in fluorescence properties. More preferably, the instant indicators display an intensity increase or decrease in emission energy upon the complexation of the desired target ion.

The specific indicator used in an assay or experiment is selected based on the desired affinity for the target ion as determined by the expected concentration range in the sample, the desired spectral properties, and the desired selectivity. Initially, the suitability of a material as an indicator of ion concentration is commonly tested by mixing a constant amount of the indicating reagent with a measured amount of the target ion under the expected experimental conditions.

Preferred indicators display a high selectivity, that is, they show a sufficient rejection of non-target ions. The interference of a non-target ion is tested by a comparable titration of the indicator with that ion. Although preferred target ions for most indicators of the present invention are Na^+, and K^+, any ion that yields a detectable change in absorption wavelengths, emission wavelengths, fluorescence lifetimes or other measurable optical property over the concentration range of interest is potentially measured using one of the indicators of this invention. Modifications to the electronic structure of the crown ether or indicator to produce an indicator having the appropriate combination of binding affinity, ion selectivity and spectral response for a wide variety of metal ions.

In one embodiment of the invention, the target ions for the indicators of the present invention are selected from Li^+, Na^+, K^+, Cs^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Rb^+, Tb^{3+} or Eu^{3+}. In another embodiment of the invention, the target ions are selected from Li^+, Na^+, K^+, Ca^{2+}, Zn^{2+}, and Mg^{2+}. Additional target ions for selected embodiments of the present indicators also include Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cu^+, Zn^{2+}, Al^{3+}, Cd^{2+}, Ag^+, Au^+, Tl^+, Pd^{2+}, Hg^{2+}, Hg^+, Sn^{2+}, Pb^{2+}, Sr^{2+}, Ba^{2+}, Mo^{3+}, Ga^{3+}, In^{3+}, La^{3+}, Eu^{3+}, Tb^{3+}, Dy^{3+}, Ru^{3+}, Sc^{3+}, As^{3+}, Sb^{3+}, Cr^{3+}, Bi^{3+}, Ce^{3+}, Ce^{4+}, Pd^{2+}, Pt^{2+} and Pt^{4+} ions. In yet another embodiment of the invention, the target ions of the instant indicators are Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cu^+, Zn^{2+}, Al^{3+}, Cd^{2+}, Hg^{2+}, Pb^{2+}, Ba^{2+}, La^{3+}, Tb^{3+}, Cr^{3+} ions. In yet another embodiment, the target ions are selected from the group consisting of Fe^{3+}, Ni^{2+}, Cu^{2+}, Cu^+, Hg^{2+}, or Pb^{2+}.

The indicator is generally prepared for use as a detection reagent by dissolving the indicator in solution at a concentration that is optimal for detection of the indicator at the expected concentration of the target ion. Modifications that are designed to enhance permeability of the indicator through the membranes of living cells, such as acetoxymethyl esters and acetates, may require the indicator to be predissolved in an organic solvent such as dimethylsulfoxide (DMSO) before addition to a cell suspension, where the indicators then readily enter the cells. Intracellular enzymes cleave the esters to the more polar acids and phenols that are then well retained inside the cells. For applications where permeability of

cell-membranes is required, the indicators of the invention are typically substituted by only one fluorophore.

A specific indicator of the present invention is useful for the detection and/or quantification of a desired target ion, when the binding of the target ion in the metal ion-binding moiety of the indicator results in a detectable change in spectral properties. Preferably, the change in spectral properties is a detectable fluorescence response.

A detectable fluorescence response, as used herein, is a change in a fluorescence property of the indicator that is capable of being perceived, either by direct visual observation or instrumentally, and the presence or magnitude of which is a function of the presence of a target metal ion in the test sample. This change in a fluorescence property is typically a change in fluorescence quantum yield, fluorescence polarization, fluorescence lifetime, a shift in excitation or emission wavelength or a combination of these effects. The detectable change in a given spectral property is generally an increase or a decrease. However, spectral changes that result in an enhancement of fluorescence intensity and/or a shift in the wavelength of fluorescence emission or excitation are also useful. The change in fluorescence on ion binding is usually due to conformational or electronic changes in the indicator that may occur in either the excited or ground state of the fluorophore, due to changes in electron density at the ion binding site, due to quenching of fluorescence by the bound target metal ion, or due to any combination of these or other effects.

A preferred indicator for a specific target ion is an indicator that shows at least a two-fold change in net fluorescence emission intensity (either higher or lower), or a 1 nanosecond difference in fluorescence lifetime (either shorter or longer), preferably a five-fold or greater change in net fluorescence emission intensity or a 100% change in fluorescence lifetime in response to the target ion. Alternatively, an indicator that exhibits a shift in excitation or emission wavelength of at least 10 nm (either to shorter or longer wavelength) is also preferred, more preferably exhibiting a shift of 25 nm or greater.

The response of an individual indicator to a specific metal ion is dependent on the

properties of indicator in the presence and absence of the target ion, the relative electron densities of the dye and metal binding site and the ability of metal ions to quench fluorescence emission when in close proximity to a fluorophore (heavy atom quenching). In one embodiment of the invention, the indicator is essentially nonfluorescent or has low fluorescence in target ion-free solution and exhibits an increase in fluorescence intensity or fluorescence lifetime (or both) upon target metal ion binding. In yet another embodiment of the invention, the fluorescence intensity remains approximately the same but there is a shift in the excitation or emission spectrum, or both, upon metal ion binding.

The optical response of the indicating reagent is determined by changes in absorbance or fluorescence, preferably fluorescence. If absorbance measurements are used to determine ion concentrations, then it is usually optimal to adjust the optical density of the indicator in the sample over the range of analyte concentration to a value of approximately 0.02 to 2.5 (most preferably 0.1 to 1). For fluorescence measurements, the concentration of the indicator will depend mostly on the sensitivity of the equipment used for its detection.

If the optical response of the indicator will be determined using fluorescence measurements, samples are typically stained with indicator concentrations of 10^{-9} M to 10^{-2} M. The most useful range of analyte concentration is about one log unit above and below the dissociation constant of the ion-indicator complex. This dissociation constant is determined by titration of the indicator with a known concentrations of the target ion, usually over the range of virtually zero concentration to approximately 100 millimolar of the target ion, depending on which ion is to be measured and which indicator is being used. The dissociation constant may be affected by the presence of other ions, particularly ions that have similar ionic radii and charge. It may also be affected by other conditions such as ionic strength, pH, temperature, viscosity, presence of organic solvents and incorporation of the sensor in a membrane or polymeric matrix, or conjugation or binding of the sensor to a protein or other biological molecule. Any or all of these effects need to be taken into account when calibrating an indicator.

The indicator is combined with a sample in a way that will facilitate detection of the target ion concentration in the sample. The sample is generally a fluid or liquid suspension that is known or suspected to contain the target ion. Representative samples include intracellular fluids such as in blood cells, cultured cells, muscle tissue, neurons and the like; extracellular fluids in areas immediately outside of cells; in vesicles; in vascular tissue of plants and animals; in biological fluids such as blood, saliva, and urine; in biological fermentation media; in environmental samples such as water, soil, waste water and sea water; in industrial samples such as pharmaceuticals, foodstuffs and beverages; and in chemical reactors. Detection and quantitation of the target ion in a sample can help characterize the identity of an unknown sample, or facilitate quality control of a sample of known origin.

In one embodiment of the invention, the sample contains cells, and the indicator is combined with the sample in such a way that the indicator is present within the sample cells. By selection of the appropriate chelating moiety, fluorophore, and the substituents thereon, indicators are prepared that will selectively localize in desired organelles, and provide measurements of the target ion in those organelles. Conjugates of the indicators of the invention with organelle-targeting peptides are used to localize the indicator to the selected organelle, facilitating measurement of target ion presence or concentration within the organelle (as described in U.S. Patent No. 5,773,227 to Kuhn et al., (1998), incorporated by reference). Alternatively, selection of a lipophilic fluorophore, or a fluorophore having predominantly lipophilic substituents will result in localization in lipophilic environments in the cell, such as cell membranes. Selection of cationic indicators will typically result in localization of the indicator in mitochondria.

In another aspect of the invention, a composition of matter comprises any of the compounds described above, and optionally includes a metal ion. In one embodiment, the compounds of the invention, in any of the embodiments described above, are associated, either covalently or noncovalently, with a surface such as a microfluidic chip, a silicon chip, a microscope slide, a microplate well, or another solid matrix, and is combined with the sample of interest as it flows over the surface. The detectable optical response is therefore detected

on the matrix surface itself, typically by use of an instrumental. This embodiment of the invention is particularly suited to high-throughput screening using automated methods.

Quantification of target ion levels in samples is typically accomplished using the indicators of the present invention by methods known in the art (Haugland, HANDBOOK OF FLUORESCENT PROBES AND RESEARCH CHEMICALS, 1996, pp., 503-544). For example, the ratiometric measurement of ion concentration provides accurate measurement of ion concentrations by the treatment of the fluorescence data as the ratio of excitation or fluorescence intensities at two wavelengths, rather than the absolute intensity at a single wavelength. Using the ratio method, a number of variables that may perturb the ion concentration measurements are eliminated. In particular, ion-dependent factors that affect the signal intensity, such as nonuniform intracellular dye concentrations, probe leakage, dye bleaching and cell thickness, are canceled in the ratio measurements, since these parameters have a similar effect on intensities at both wavelengths. While the ratio method can be used to determine concentrations using observation of either the excitation spectra of the indicator, the emission spectra of the indicator, or both, in the case of the indicators of the present invention, the shift in excitation energy upon binding metal ions makes observation of the excitation spectrum a more useful technique. In either case, to achieve maximal utility, the indicator must be calibrated (to compensate for variance in the dissociation constant of the indicator due to ionic strength, viscosity, or other conditions within the sample). To calibrate the indicator, ionophores such as A-23187, gramicidin, valinomycin, or ionomycin are used. Non-ratiometric analysis can also be accomplished by calibration with a second fluorescent dye present in the sample.

The optical response of the indicator to the ion can be detected by various means that include measuring absorbance or fluorescence changes with an instrument, visually, or by use of a fluorescence sensing device. Several examples of fluorescence sensing devices are known, such as fluorometers, fluorescence microscopes, laser scanners, flow cytometers, and microfluidic devices, as well as by cameras and other imaging equipment. These measurements may be made remotely by incorporation of the fluorescent ion sensor as part of a fiber optic probe. The indicator is covalently attached to the fiber optic probe material,

typically glass or functionalized glass (e.g., aminopropyl glass) or the indicator is attached to the fiber optic probe via an intermediate polymer, such as polyacrylamide. The indicator solution is alternatively incorporated non-covalently within a fiber optic probe, as long as there is a means whereby the target ion can come into contact with the indicator solution.

Kits

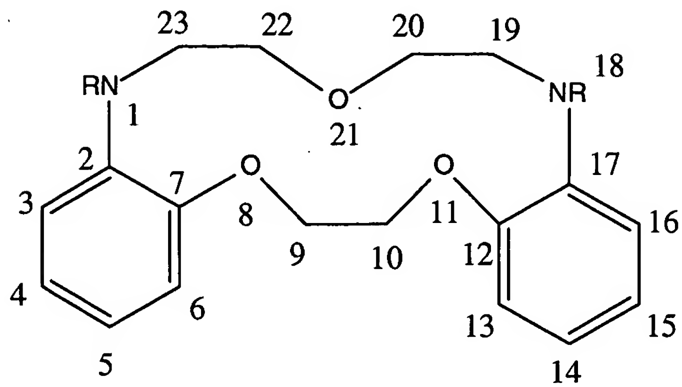
Due to the advantageous properties and the simplicity of use of the instant crown ether compounds, they are particularly useful in the formulation of a kit for the complexation, detection, or quantification of selected target ions, comprising one or more compounds or compositions of the invention in any of the embodiments described above (optionally in a stock solution), instructions for the use of the crown ether compound to complex or detect a desired target ion, and optionally comprising additional components. In one aspect, the compounds of the invention are associated with a surface, such as a chip, microplate well, or other solid matrix, and the sample of interest flows over the surface. The detectable optical response is therefore detected on the matrix surface itself.

The additional kit components may be selected from, without limitation, calibration standards of a target ion, ionophores, fluorescence standards, aqueous buffers, and organic solvents. The additional kit components are present as pure compositions, or as aqueous solutions that incorporate one or more additional kit components. Any or all of the kit components optionally further comprise buffers.

The examples below are given so as to illustrate the practice of this invention. They are not intended to limit or define the entire scope of this invention.

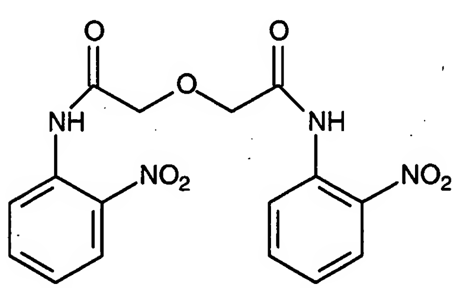
EXAMPLES

The following numbering scheme is used throughout the following examples:



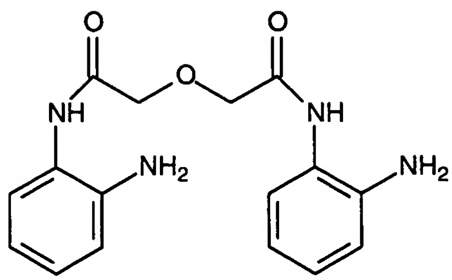
Example 1. Preparation of a tetraaza-crown ether.

2-Nitroaniline is acylated with diglycolyl chloride in anhydrous THF, using 0.5 equivalents of diglycolyl chloride in dilute solution. A 0.1 M THF solution of diglycolyl chloride is slowly added to a 0.1 M THF solution of 2-nitroaniline, containing two equivalents of triethylamine. After TLC indicates consumption of all starting 2-nitroaniline, volatiles are removed in vacuo and the residue is partitioned between water and ethyl acetate. The ethyl acetate layer is concentrated to give Compound **1**.



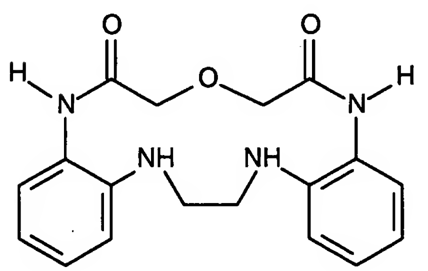
Compound **1**

Compound **1** is dissolved in methanol and treated with 20% by weight of 10% palladium on carbon. The resulting mixture is shaken under 40 psi hydrogen until analysis by thin layer chromatography (TLC) indicates the reaction is complete. The reaction mixture is filtered and the bis-aniline **2** is isolated by concentration *in vacuo* of the filtrate.



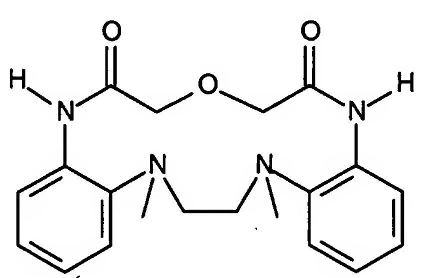
Compound **2**

Compound **2** is dissolved in DMF to achieve a 0.1 M solution, then treated with 0.9 equivalents of 1,2-dibromoethane, two equivalents of diisopropylethylamine, and catalytic sodium iodide. The resulting solution is heated until most of **2** is consumed, as judged by TLC analysis. The volatiles are removed *in vacuo*, and the residue is partitioned between ethyl acetate and water. The ethyl acetate layer is concentrated *in vacuo* to give compound **3**.



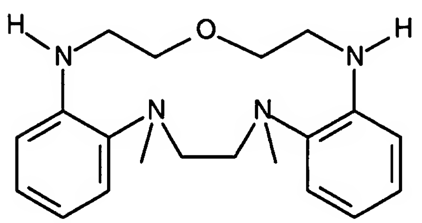
Compound **3**

Compound **3** is dissolved in acetonitrile to achieve a 0.5 M concentration. Ten equivalents of formaldehyde, as a 37% aqueous solution, are added followed by 4 equivalents of sodium cyanoborohydride. The pH of the reaction mixture is adjusted to 7 with acetic acid, and the resulting solution is stirred until TLC analysis indicates consumption of **3** and formation of **4**. Volatiles are removed *in vacuo*, and the residue is partitioned between ethyl acetate and water. The ethyl acetate layer is concentrated in vacuo to give compound **4**.



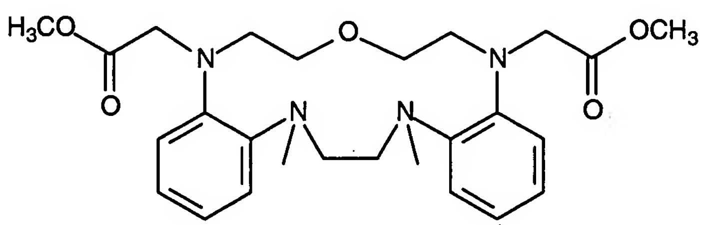
Compound **4**

Compound **4** is dissolved in THF at 1 M concentration, and treated with 10 equivalents of borane-THF (1M). The resulting solution is heated at reflux until **4** is consumed and **5** is formed, as judged by TLC analysis. The reaction is quenched by addition of 5% aqueous sodium hydroxide, followed by extraction with ethyl acetate. The ethyl acetate layer is concentrated in vacuo to give **5**.



Compound **5**

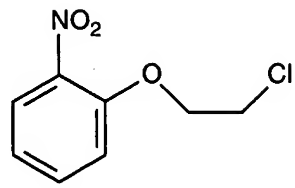
Compound **5** is dissolved in DMF at 0.5 M concentration. Ten equivalents of methyl bromoacetate and 4 equivalents of diisopropylethylamine are added, and the resulting solution is stirred at 100 degrees centigrade until TLC analysis indicates consumption of **5** and formation of **6**. Volatiles are removed in vacuo, and the residue is partitioned between water and ethyl acetate. The ethyl acetate layer is concentrated in vacuo to give **6**, which can be further purified by flash chromatography on silica gel using methanol in chloroform as eluant.



Compound **6**

Example 2. Preparation of (2'-Nitrophenoxy)-2-chloroethane (**102**).

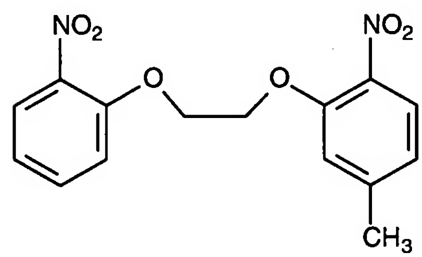
A suspension of 2-nitrophenol **101** (50.0 g, 0.36 mol), 1-bromo-2-chloroethane (45 mL, 0.54 mol), and K_2CO_3 (100.0 g, 0.72 mol) in DMF (200 mL) was stirred at 90°C for 2 h, cooled to room temperature, poured into ice-water, filtered, washed with H_2O and dried to give Compound **102,** 70.3 g (96%) as a yellow solid.



Compound **102**

Example 3. Preparation of 1-(5'-methyl-2'-nitrophenoxy)-2-(2''-nitrophenoxy)ethane (**104**).

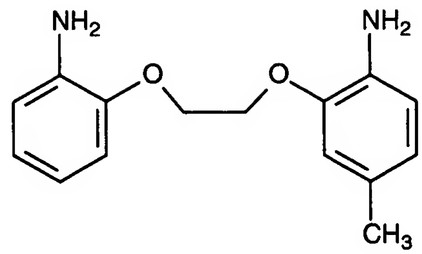
A suspension of Compound **102** (60.45 g, 0.30 mol), 5-methyl-2-nitrophenol (50.49 g, 0.33 mol), and K_2CO_3 (82.80 g, 0.60 mol) in DMF (450 mL) was stirred at 130 °C for 18 h, cooled to room temperature, poured into ice-water, filtered, washed with H_2O and dried to give Compound **104,** 89.50 g (93.5%) as an orange solid.



Compound **104**

Example 4. Preparation of 1-(2'-amino-5'-methylphenoxy)-2-(2''-aminophenoxy)ethane (**105**).

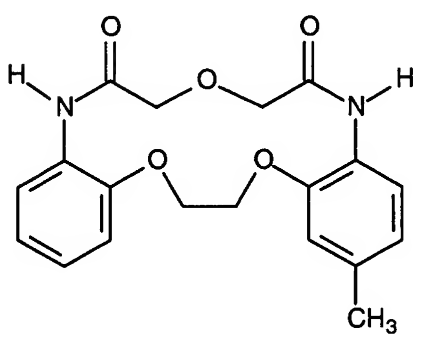
Compound **104** (24.0 g, 75 mmol) was hydrogenated over 10% Pd/C (1.5 g) in DMF (250 mL) at 40 psi for 18 h. The mixture was filtered from catalyst through a CELITE pad on a fritted glass filter. The filtrate was evaporated and ether (50 mL) was added. The product was filtered, washed with ether and dried to give compound **105,** 18.0 g (95%) as a brown solid.



Compound **105**

Example 5. Preparation of 5-methyl-19,23-dioxo-DDTCPD (**106**).

To a stirred mixture of diamine **105** (13.10 g, 50 mmol) and Et_3N (20.8 mL, 150 mmol) in dry THF (2.5 L) was slowly added over 12 h a solution of diglycolyl dichloride (6.3 mL, 55 mmol) in dry THF (0.5 L). The reaction mixture was stirred for 6 h, filtered from the precipitated hydrochloride, and washed with THF. The combined organic filtrate was evaporated. The residue was dissolved in $CHCl_3$ (800 mL) and washed successively with 0.5 M HCl, H_2O, saturated $NaHCO_3$ then saturated NaCl. The organic layer was dried over $MgSO_4$ and evaporated. Ether (100 mL) was added and the precipitated product was filtered, washed with ether and dried to give Compound **106**, 16.0 g (90%) as an off-white solid.

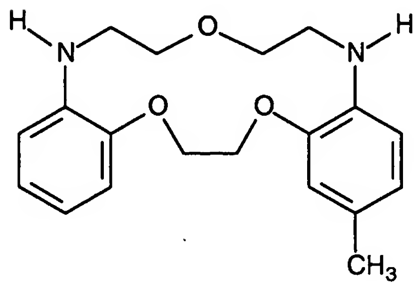


Compound **106**

Example 6. Preparation of 5-methyl-DDTCPD (**107**).

To a suspension of diamide **106** (15.60 g, 44 mmol) in dry THF (400 mL) was added a 1 M solution of BH_3-THF complex in dry THF (390 mL, 390 mmol). The mixture was stirred at 70 °C for 16 h. Dry methanol (300 mL) was added dropwise over 1 h into a boiling mixture with strong gas evaluation. The mixture was heated at reflux for 2 h, cooled to room

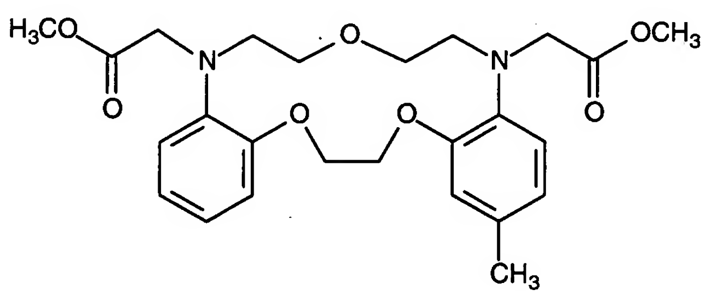
temperature, evaporated and co-evaporated with MeOH to destroy the borane complex. Ether (100 mL) was added. The solid product was filtered, washed with ether and dried to give Compound **107**, 12.3 g (85%) as an off-white solid.



Compound **107**

Example 7. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-methyl-DDTCPD (**108**).

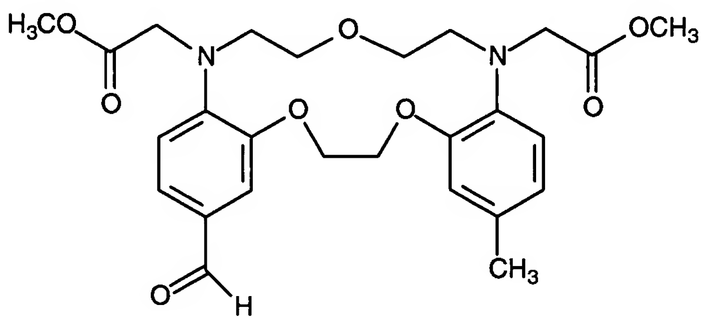
A mixture of diamine **107** (6.56 g, 20 mmol), methyl bromoacetate (38 mL, 400 mmol), diisopropylethylamine (DIPEA) (104 mL, 600 mmol), and NaI (1.50 g, 10 mmol) in MeCN (300 mL) was heated at reflux for 70 h. After cooling to room temperature, the MeCN was evaporated. The residue was dissolved in $CHCl_3$, washed with 1% AcOH then H_2O, dried and evaporated. The residual oily product was cooled to 0 °C and washed with cold hexane to remove most of the alkylating reagent. The crude product **108** was used immediately in the next step without further purification.



Compound **108**

Example 8. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-formyl-14-methyl-DDTCPD (**109**).

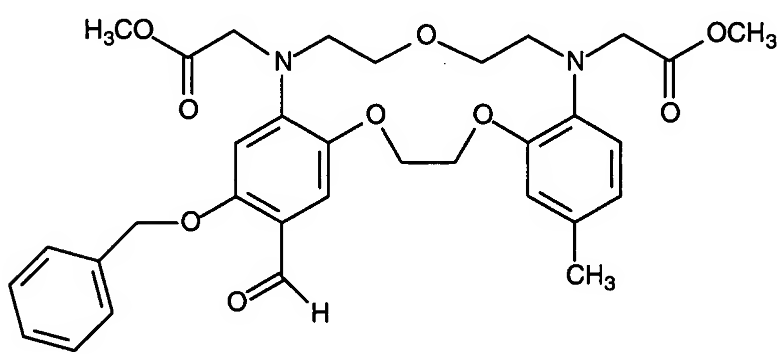
To a stirred solution of the Vilsmeier reagent prepared from $POCl_3$ (4.65 mL, 50 mmol) and 30 mL DMF was added over 5 min a solution of Compound **108** (4.8 g, 10 mmol) in DMF (20 mL). The mixture was stirred for 16 h, cooled by the addition of ice and neutralized with saturated K_2CO_3 to pH 7-8. The suspension was extracted with $CHCl_3$, washed successively with 0.1 M HCl, saturated NaCl, saturated $NaHCO_3$ and saturated NaCl, then evaporated. The residue was dissolved in $CHCl_3$ and chromatographed on SiO_2 with an EtOAc gradient in hexanes (20% to 30%) to give aldehyde **109**, 2.60 g (52%) as colorless crystals.



Compound **109**

<u>Example 9. Preparation of 1,18-bis(methoxycarbonylmethyl)-4-benzyloxy-5-formyl-14-methyl-DDTCPD (**117**):</u>

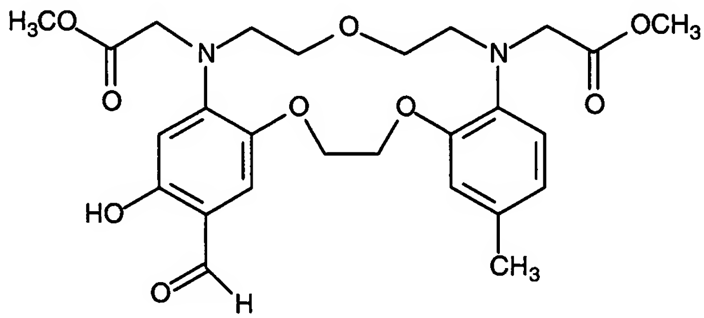
Compound **117** was prepared analogously with Compound **109**, starting with 5-methyl-2-nitrophenol in place of 2-nitrophenol, and utilizing 4-benzyloxy-2-nitrophenol in place of 5-methyl-2-nitrophenol.



Compound **117**

Example 10. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-formyl-4-hydroxy-14-methyl-DDTCPD (118).

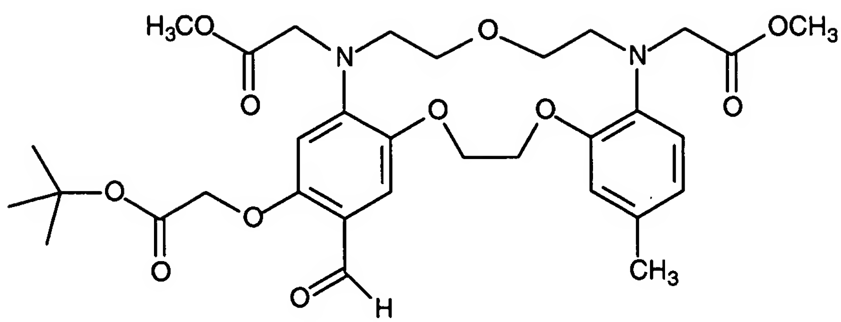
Compound **117** (3.06 g, 5.0 mmol) was hydrogenated over 10% Pd/C (0.50 g) in AcOH (100 mL) at 50 psi for 5 h. The mixture was filtered through a CELITE pad on a fritted glass filter. The filtrate was evaporated, ether (50 mL) was added, the product was filtered, washed with ether and dried to give Compound **118,** 2.13 g (82%) as a colorless solid.



Compound **118**

Example 11. Preparation of 1,18-bis(methoxycarbonylmethyl)-4-*t*-butoxycarbonylmethoxy-5-formyl-14-methyl-DDTCPD (119).

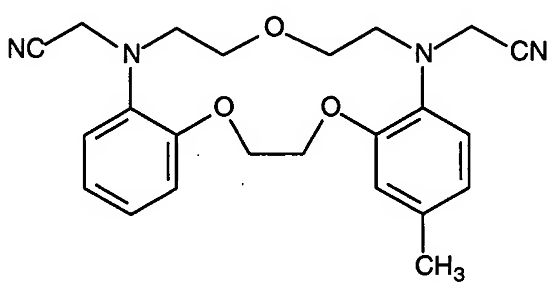
To a suspension of Compound **118** (0.800 g, 1.55 mmol), K_2CO_3 (1.04 g, 7.5 mmol) and NaI (0.03 g, 0.2 mmol, catalyst) in DMF (10 mL) was added dropwise *t*-butyl bromoacetate (0.45 mL, 3.0 mmol). The mixture was stirred for 16 h, diluted with H_2O, extracted with $CHCl_3$, dried over $MgSO_4$ and evaporated. The residue was dissolved in hexane: EtOAc (1:1) and chromatographed on silica gel with 40% EtOAc in hexanes to give aldehyde **119**, 0.875 g (90%) as colorless crystals.



Compound **119**

Example 12. Preparation of 1,18-bis(cyanomethyl)-5-methyl-DDTCPD (**120**).

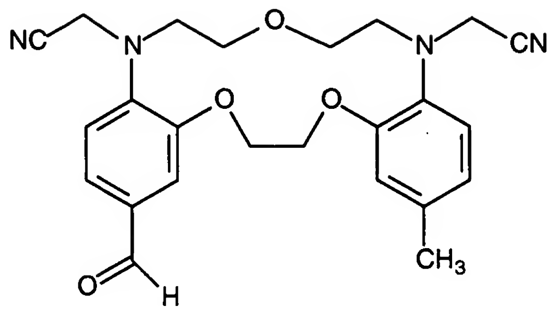
A stirred mixture of diamine **107** (3.38 g, 10 mmol), bromoacetonitrile (14 mL, 200 mmol), DIPEA (26 mL, 150 mmol), NaI (1.5 g, 10 mmol; catalyst) in MeCN (150 mL) was heated at reflux for 70 h. After cooling to room temperature the MeCN was evaporated. The residue was dissolved in $CHCl_3$ (800 mL), washed with H_2O, dried over $MgSO_4$ and evaporated. The residue was chromatographed on SiO_2 with 40% EtOAc in hexanes to give Compound **120**, 3.56 g (90%) as a colorless solid.



Compound **120**

Example 13. Preparation of 1,18-bis(cyanomethyl)-5-formyl-14-methyl-DDTCPD (**121**).

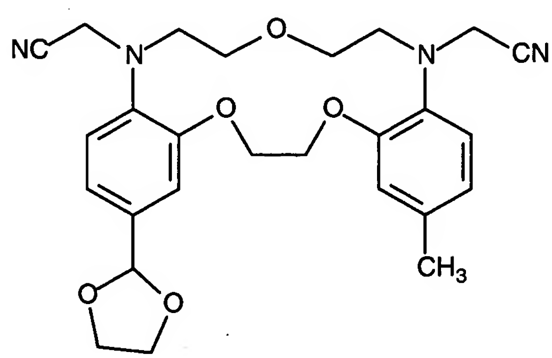
To a stirred solution of the Vilsmeier reagent prepared from $POCl_3$ (9.5 mL, 102 mmol) and 60 mL DMF was added over 5 min to a solution of Compound **120**. The mixture was stirred for 100 h at 40 °C, cooled by the addition of ice and neutralized with saturated K_2CO_3 to pH 8. The suspension was extracted with $CHCl_3$, washed with H_2O, dried over $MgSO_4$ and evaporated. The residue was chromatographed over SiO_2 using $CHCl_3$ as eluant to give compound **121**, 3.12 g (73%) as colorless crystals.



Compound **121**

Example 14. Preparation of 1,18-bis(cyanomethyl)-5-(2',5'-dioxacyclopentyl)-14-methyl-DDTCPD (122).

A mixture of aldehyde **121** (3.08 g, 7.3 mmol), ethylene glycol (8 mL, 140 mmol), p-toluenesulphonic acid (0.20 g, catalyst) and benzene (80 mL) was refluxed with a Dean-Stark trap for 4 h. After cooling to room temperature the mixture was evaporated. The residue was dissolved in $CHCl_3$ (300 mL), washed with saturated $NaHCO_3$, dried over $MgSO_4$, and evaporated to give compound **122**, 2.82 g (85%) as an orange solid. Compound **122** was pure on TLC and used in the next step without additional purification.

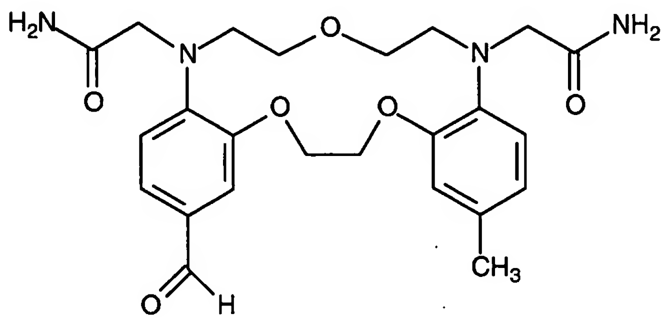


Compound **122**

Example 15. Preparation of 1,18-bis(carbamoylmethyl)-5-formyl-14-methyl-DDTCPD (123).

To a solution of Compound **122** (0.200 g, 0.44 mmol) in MeOH (25 mL) and 1 M NaOH (15 mL, 15 mmol) was added over 5 min 30% H_2O_2 (5 mL, catalyst). The mixture was stirred for 1 h, then acidified with 1 M HCl to pH 1. The acidified mixture was stirred for 30 min,

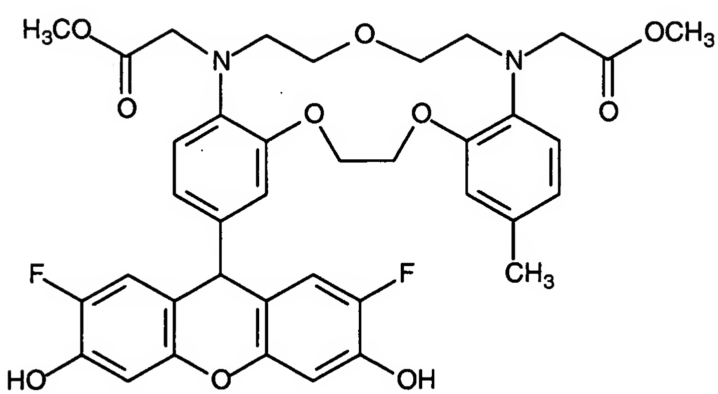
diluted with 3 M NaOAc (150 mL), extracted with $CHCl_3$, washed with saturated $NaHCO_3$, dried over $MgSO_4$ and evaporated to give aldehyde **123,** 0.191 g (95%) as a colorless crystalline material, pure by TLC.



Compound **123**

Example 16. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-fluoro]-DDTCPD (131).

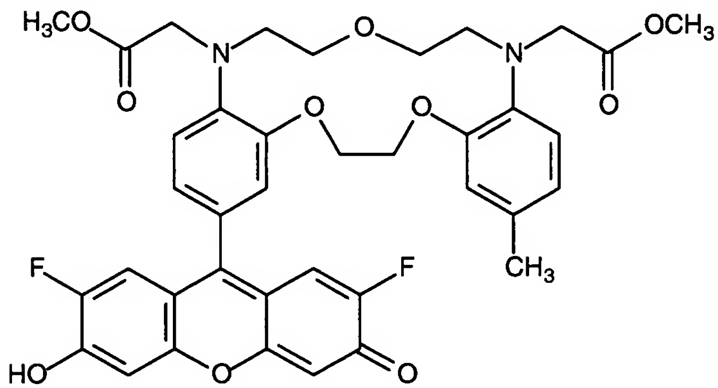
A mixture of aldehyde **109** (1.150 g, 2.3 mmol) and 4-fluororesorcinol (0.670 g, 5.2 mmol) in $MeSO_3H$ (25 mL) was stirred overnight, then poured into 3 M NaOAc (300 mL). The precipitated solid was filtered, washed with H_2O and dried to give compound **131**, 1.605 g (97%) as an off-white solid. Compound **131** was unstable to oxidation and was used in the next step without additional purification.



Compound **131**

Example 17. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[fluoro]-DDTCPD (132).

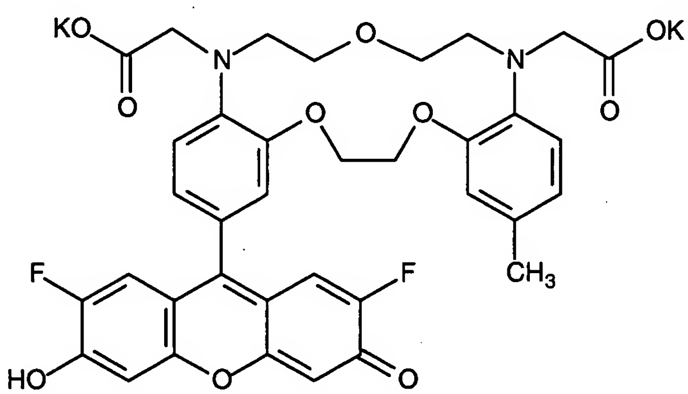
A mixture of Compound **131** (150 mg, 0.21 mmol) and chloranil (246 mg, 1.0 mmol) in MeOH (5 mL) and $CHCl_3$ (5 mL) was refluxed for 50 h, then cooled to room temperature, filtered from excess oxidizer, and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using $CHCl_3$: MeOH:AcOH (20:2:1) to give compound **132**, 44 mg (20%) as a red-brown solid.



Compound **132**

<u>Example 18. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[fluoro]-DDTCPD (133).</u>

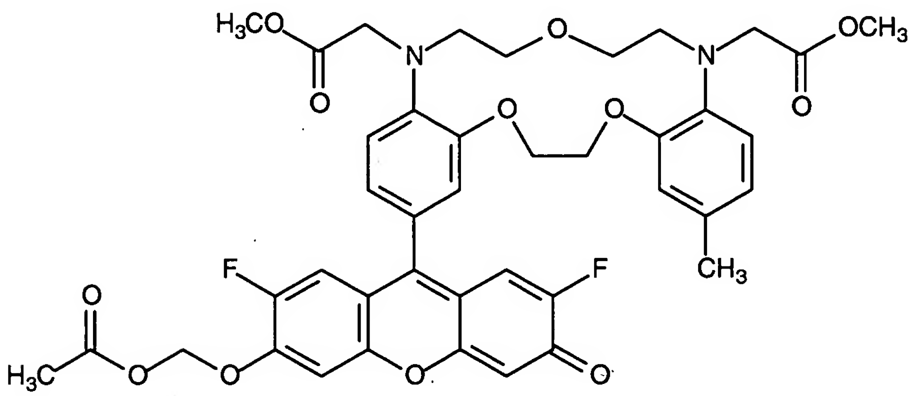
A mixture of Compound **132** (30 mg, 0.04 mmol) in MeOH (5 mL) and 1 M KOH (1 mL, 1.0 mmol) was stirred for 16 h, then neutralized with 1 M HCl to pH 7, and evaporated to dryness. The residue was purified by preparative TLC on two SiO_2 plates using $CHCl_3$: MeOH:AcOH (13:3:1) to give compound **133**, 21 mg (74%) as a brown solid.



Compound **133**

Example 19. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[fluoro-AM]-DDTCPD (134).

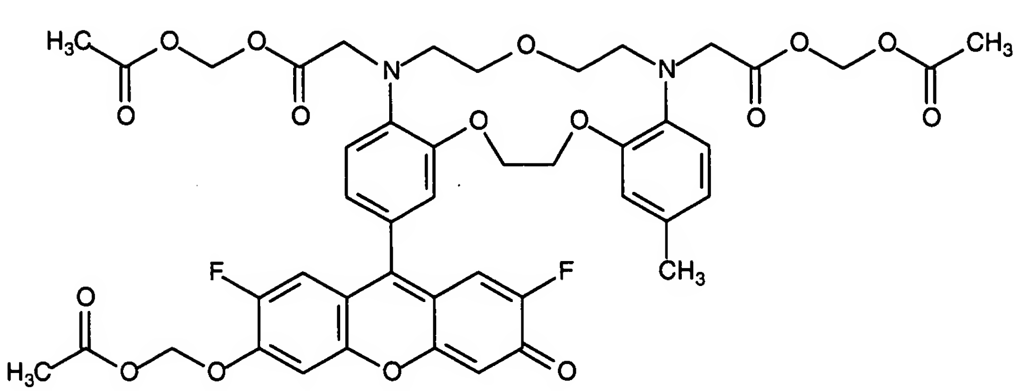
A mixture of Compound **132** (78 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.175 mL, 1 mmol) in DMF (1 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension was extracted with $CHCl_3$, washed with H_2O, filtered and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$: to give compound **134**, 25 mg (32%) as a brown solid.



Compound **134**

Example 20. Preparation of 1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[fluoro-AM]-DDTCPD (135).

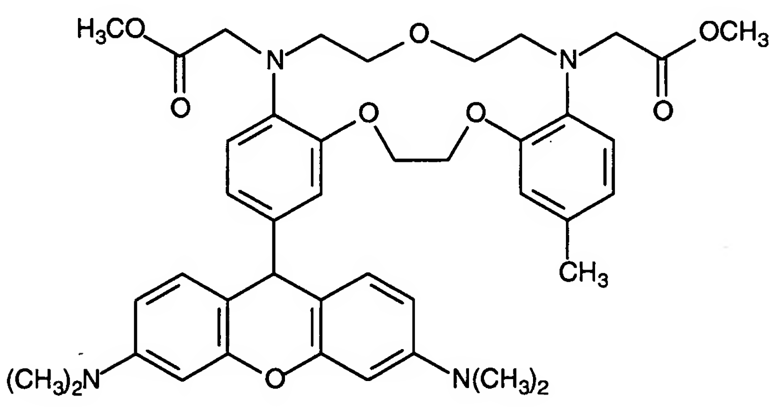
A mixture of Compound **133** (69 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.175 mL, 1 mmol) in DMF (1 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension was extracted with $CHCl_3$, washed with H_2O, filtered and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give Compound **135** as a brown solid.



Compound **135**

Example 21. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-TMR]-DDTCPD (137).

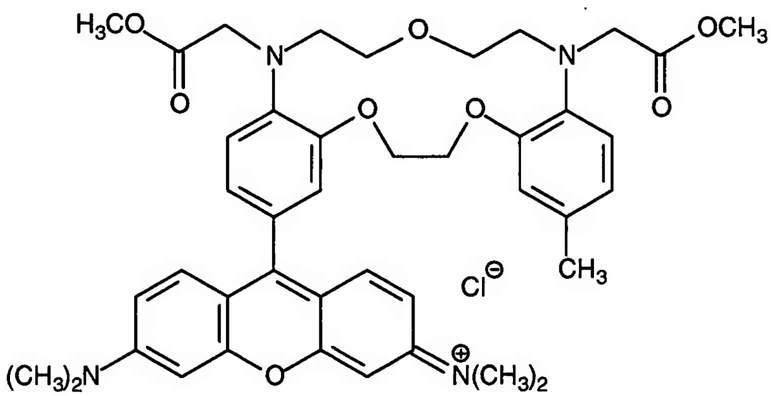
A mixture of aldehyde **109** (250 mg, 0.5 mmol), 3-dimethylaminophenol (157 mg, 1.1 mmol), and p-toluenesulphonic acid (10 mg, catalyst) in propionic acid (5 mL) was stirred overnight at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (100 mL). The precipitated solid was filtered, washed with H_2O and dried to give compound **137,** 360 mg (97%) as a rose-colored solid. Compound **137** was unstable to oxidation and was used in subsequent reactions without additional purification.



Compound **137**

Example 22. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (138).

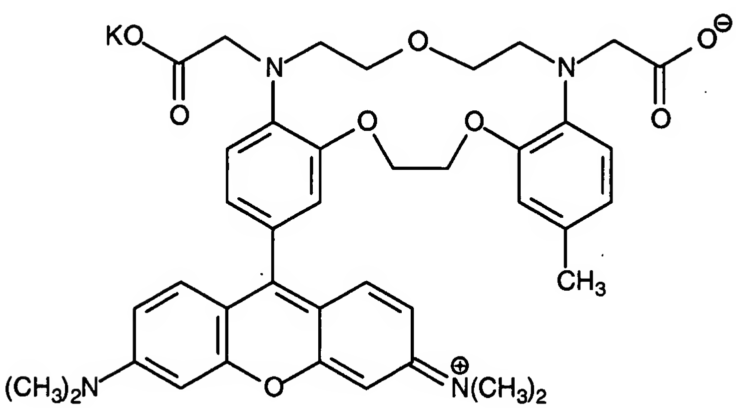
A mixture of Compound **137** (660 mg, 0.81 mmol) and chloranil (400 mg, 1.62 mmol) in MeOH (25 mL) and $CHCl_3$ (25 mL) was stirred for 2 h, filtered from excess oxidizer, and evaporated. The residue was purified by chromatography on SiO_2 using 9% MeOH and 1% AcOH in $CHCl_3$ as eluant to give the crude product, which was again chromatographed on SiO_2 using the same eluant to give Compound **138,** 112 mg (19%) as crimson solid.



Compound **138**

<u>Example 23. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[TMR]-DDTCPD potassium salt (139).</u>

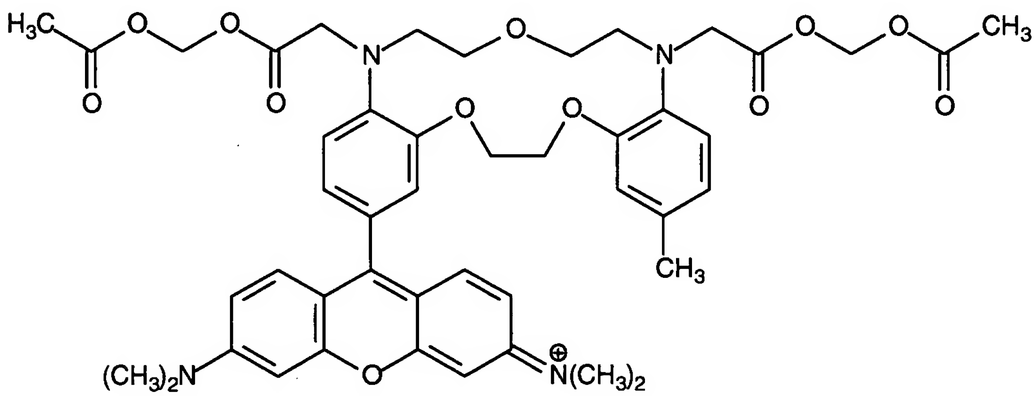
A mixture of Compound **138** (40 mg, 0.05 mmol) in MeOH (2 mL) and 1 M KOH (1 mL, 1.0 mmol) was stirred for 16 h, then evaporated to dryness. The residue was purified by chromatography on a SEPHADEX LH-20 column using H_2O as eluant to give salt **139**, 8 mg (21%) as red flakes after lyophilization.



Compound **139**

Example 24. Preparation of 1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (**140**).

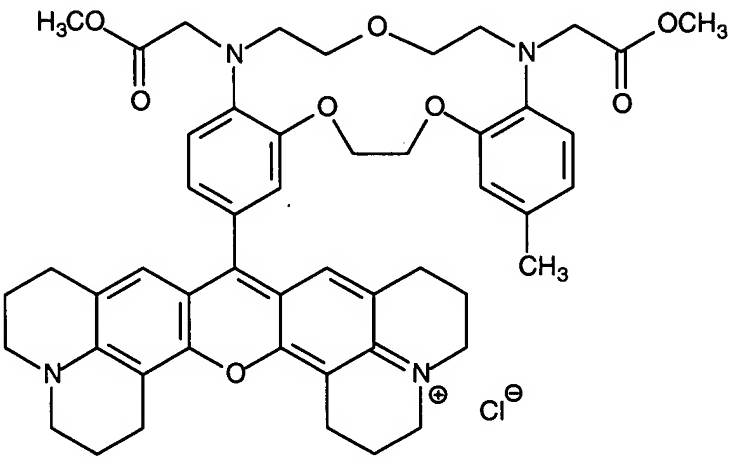
A mixture of Compound **139** (75 mg, 0.1 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.35 mL, 2 mmol) in DMF (2 mL) was stirred for 2 h, then poured into 1% AcOH (200 mL). The suspension is extracted with $CHCl_3$, washed with H_2O, filtered and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give Compound **140** as a red solid.



Compound **140**

Example 25. Preparation of 1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[X-Rhd]-DDTCPD (**143**).

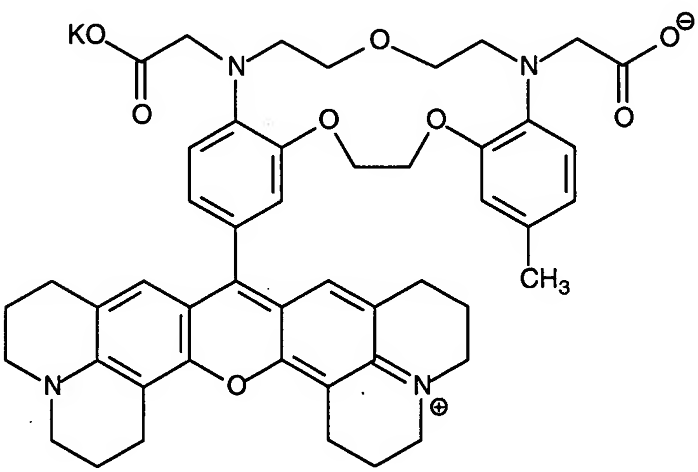
Compound **143** was prepared analogously to Compound **138**, using 8-hydroxyjulolidine rather than *m*-dimethylaminophenol.



Compound **143**

Example 26. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[X-Rhd]-DDTCPD potassium salt (144).

Compound **144** was prepared form Compound **138** using the procedure for preparing Compound **139** from Compound **138**.

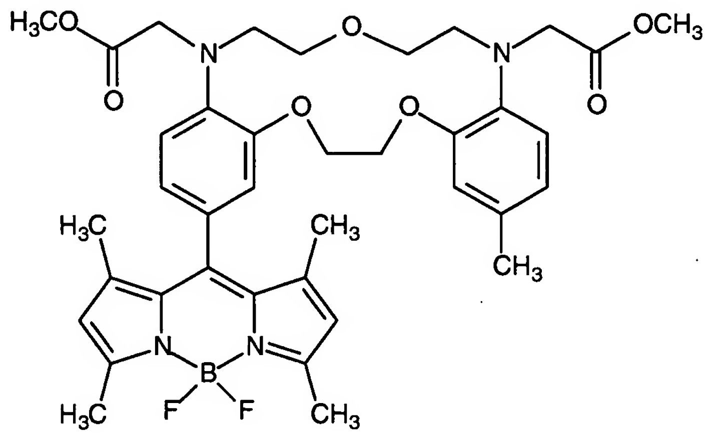


Compound **144**

Example 27. Preparation of 1,18-bis(carboxymethyl)-14-methyl-5-[BODIPY]-DDTCPD (145).

To a solution of aldehyde **109** (250 mg, 0.5 mmol) in CH_2Cl_2 (20 mL) was added 2,4-dimethylpyrrole (0.125 mL, 1.2 mmol). The solution was stirred for 5 min, then TFA (0.046

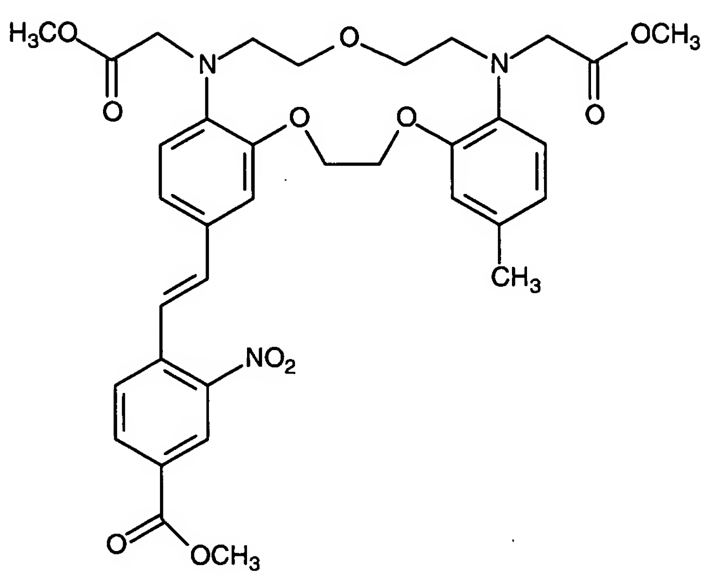
mL, 0.6 mmol) is introduced. After 16 h, the mixture was diluted with $CHCl_3$ (150 mL), washed with 2% tetrabutylammonium hydroxide (150 mL) then H_2O then evaporated. The residue was dissolved in toluene (20 mL) and stirred for 3 h with chloranil (148 mg, 0.6 mmol). DIPEA (0.87 mL, 5 mmol) was introduced, followed by BF_3 etherate (0.52 mL, 4 mmol). The mixture was evaporated and the residue was purified by chromatography on SiO_2 using a gradient of 0-2% MeOH in $CHCl_3$ to give compound **145**, 102 mg (28%) as a brown solid.



Compound **145**

Example 28. Preparation of trans 1-[1',18'-bis-(methoxycarbonylmethyl)-14'-methyl-DDTCPD-5-yl]-2-[4''-methoxycarbonyl-2''-nitrophenyl] ethylene (**147**).

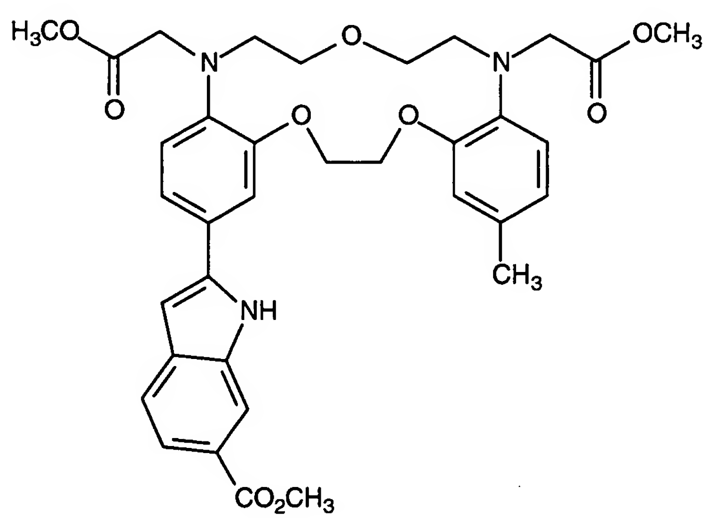
A mixture of aldehyde **109** (1.00 g, 2.0 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (1.34 g, 2.5 mmol), and K_2CO_3 (1.38 g, 10 mmol) in DMF (20 mL) was stirred for 16 h at 95 °C. More of the Wittig base (0.500 g, 0.93 mmol) was added and the mixture was stirred for an additional 6 h, then cooled to room temperature and poured into H_2O. The solution was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$, dried over $MgSO_4$, and evaporated. The crude product was purified by chromatography on SiO2 using $CHCl_3$ then 2% MeOH in $CHCl_3$ to give compound **147**, 0.915 g (68%) as an orange solid.



Compound **147**

Example 29. Preparation of 1,18-bis(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (148).

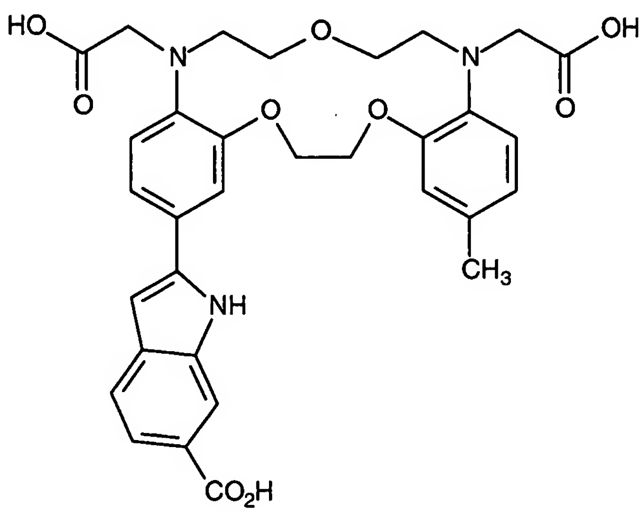
A mixture of the ethylene derivative **147** (70 mg, 0.1 mmol) and triethylphosphite (3 mL) was heated at 120 °C for 6 h, then evaporated and subsequently co-evaporated with DMF (3 x 10 mL). The residue was purified by preparative TLC on two SiO_2 plates using 7% MeOH in $CHCl_3$ as eluant to give compound **148**, 52 mg (80%) as a slightly yellowish solid.



Compound **148**

Example 30. Preparation of 1,18-bis(carboxymethyl)-5-(6'-carboxy indolyl-2')-14-methyl-DDTCPD (**149**).

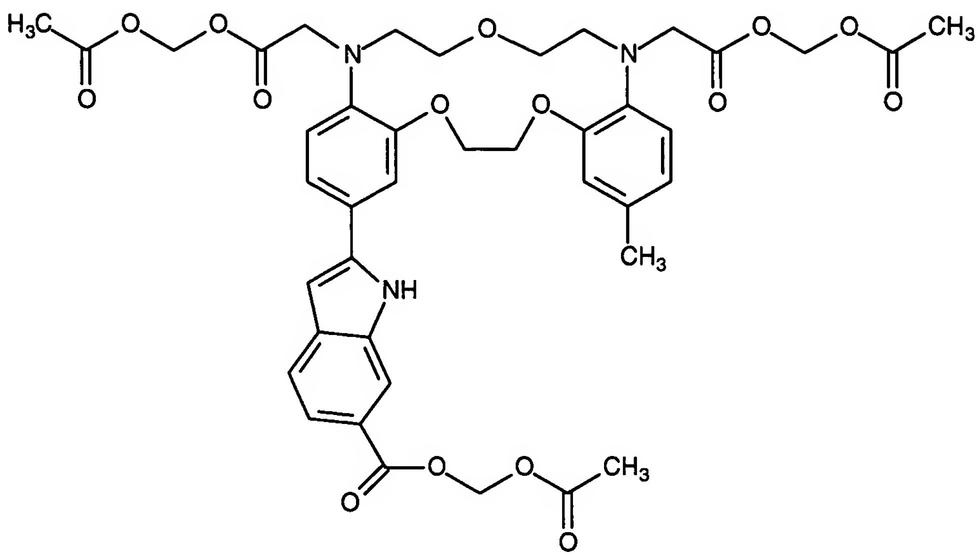
A mixture of Compound **148** (300 mg, 0.46 mmol), and 1 M KOH (6 mL, 6 mmol) in MeOH (25 mL) was stirred for 16 h. More 1 M KOH (5 mL, 5 mmol) was added and the mixture was stirred for an additional 6 h, then evaporated to half its original volume, and acidified with 1 M HCl to pH 3. The suspension was extracted with $CHCl_3$ then with n-BuOH. The combined organic extract was filtered and was evaporated. Ether (20 mL) was added and the precipitated solid was filtered and washed with ether to give compound **149**, 157 mg (57%) as an off-white solid.



Compound **149**

Example 31. Preparation of 1,18-bis(acetoxymethoxycarboxymethyl)-5-(6'-acetoxymethoxycarboxyindolyl-2')-14-methyl-DDTCPD (**150**).

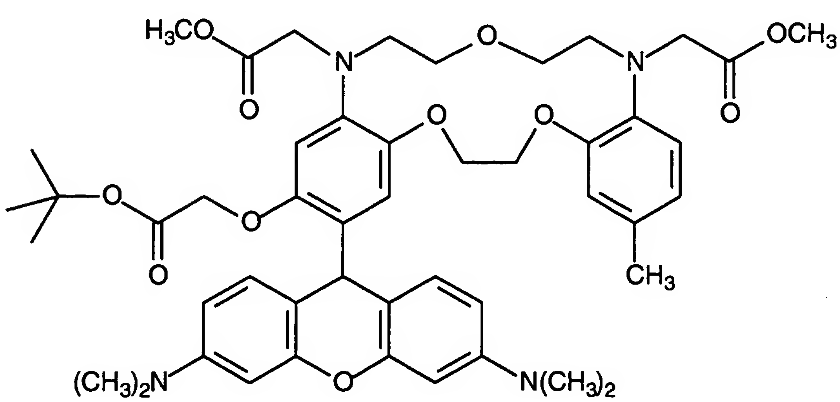
A mixture of Compound **149** (72 mg, 0.12 mmol), bromomethyl acetate (0.14 mL, 1.0 mmol), and DIPEA (0.35 mL, 2 mmol) in DMF (5 mL) was stirred for 16 h, poured into 1% AcOH (200 mL), extracted with $CHCl_3$, washed with H_2O then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give Compound **150**, 3 mg (3%) as a brown solid.



Compound **150**

Example 32. Preparation of 4-(*t*-butoxycarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[2H-TMR]-DDTCPD (**151**).

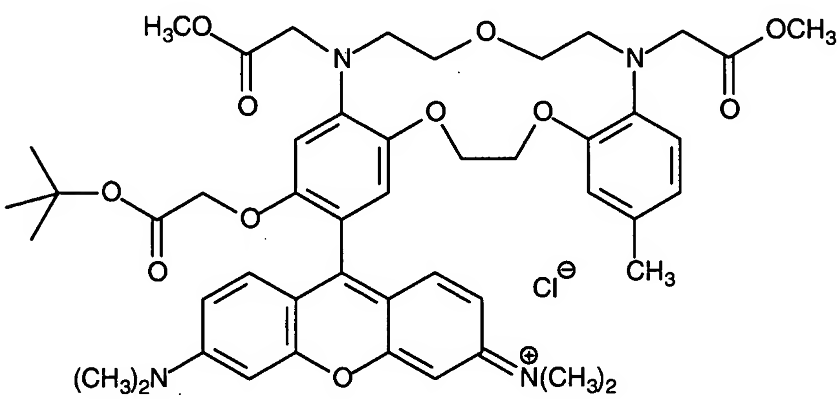
A mixture of aldehyde **119** (630 mg, 1.0 mmol), 3-dimethylaminophenol (330 mg, 2.4 mmol), and p-toluenesulphonic acid (20 mg, catalyst) in propionic acid (5 mL) was stirred for 16 h at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (150 mL). The precipitated solid was filtered, washed with H_2O and dried to give Compound **151,** 360 mg (97%) as a rose solid. Compound **151** is unstable to oxidation and was used in next step without additional purification.



Compound **151**

Example 33. Preparation of 4-(*t*-butoxycarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (**152**).

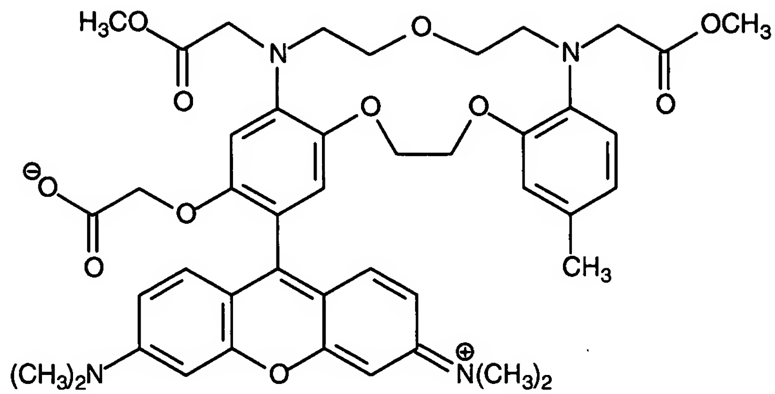
A mixture of Compound **151** (2.50 g, 2.9 mmol) and chloranil (1.42 g, 5.8 mmol) in MeOH (100 mL) and $CHCl_3$ (100 mL) was stirred for 4 h, filtered from excess oxidizer, and evaporated. The residue was purified by chromatography on SiO_2 using a gradient of 6-10% MeOH and 1% AcOH in $CHCl_3$ to give compound **152,** 1.13 g (45%) as a crimson solid.



Compound **152**

Example 34. Preparation of 4-carboxymethyloxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (**153**).

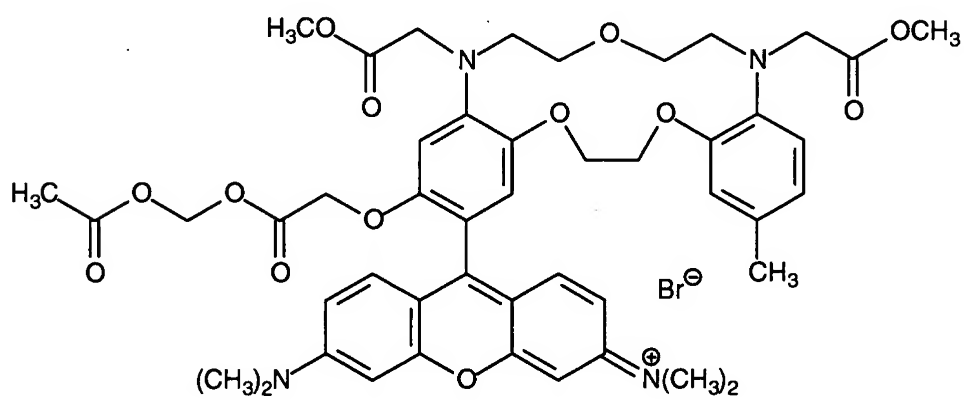
A mixture of Compound **152** (500 mg, 0.58 mmol) in CH_2Cl_2 (20 mL) and TFA (20 mL) was stirred for 4 h, then evaporated and co-evaporated with $CHCl_3$. Ether (25 mL) was added to the residue and the precipitated product was filtered and washed with ether to give Compound **153**, 447 mg (95%) as a violet-red solid.



Compound **153**

Example 35. Preparation of 4-acetoxymethoxycarbonylmethyloxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (154).

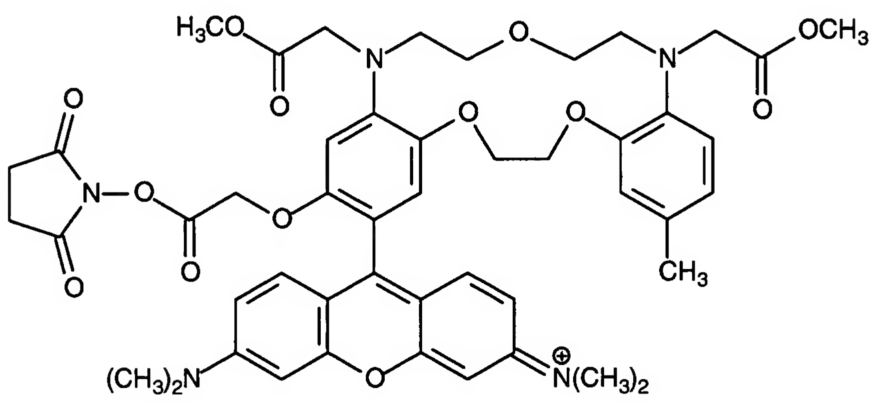
A mixture of Compound **153** (20 mg, 0.1 mmol), bromomethyl acetate (0.025 mL, 0.25 mmol), and DIPEA (0.08 mL, 0.5 mmol) in DMF (1 mL) was stirred for 3 h. More bromomethyl acetate (0.025 mL, 0.25 mmol) and DIPEA (0.08 mL, 0.5 mmol) were added and the mixture was stirred for an additional 3 h, diluted with $CHCl_3$ (100 mL), washed with 1% AcOH then H_2O then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 10% MeOH and 0.5% AcOH in $CHCl_3$ to give Compound **154**, 12 mg (54%) as a dark red solid.



Compound **154**

Example 36. Preparation of 4-carboxymethyloxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, succinimidyl ester (155).

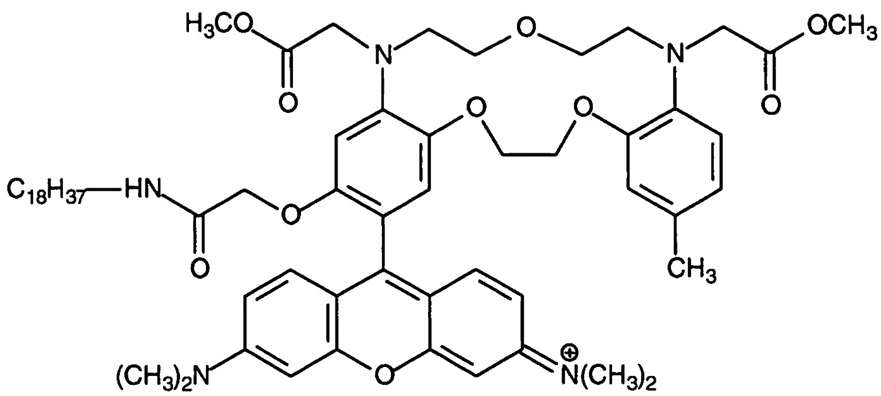
To a stirred solution of Compound **153** (162 mg, 0.2 mmol) and pyridine (0.08 mL, 1 mmol) in DMF (5 mL), was added dry N-trifluoroacetoxysuccinimide (90 mg, 0.4 mmol). After 7 h more N-trifluoroacetoxysuccinimide (90 mg, 0.4 mmol) and pyridine (0.08 mL, 1 mmol) were added, and the mixture was stirred for 16 h more. Analytical TLC confirmed the formation of the single product, while starting material **153** was consumed. Compound **155** was very reactive and unstable towards isolation attempts. It was used in further transformations upon preparation in DMF solution without isolation and purification.



Compound **155**

<u>Example 37. Preparation of 4-(N-hexadecylaminocarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (156).</u>

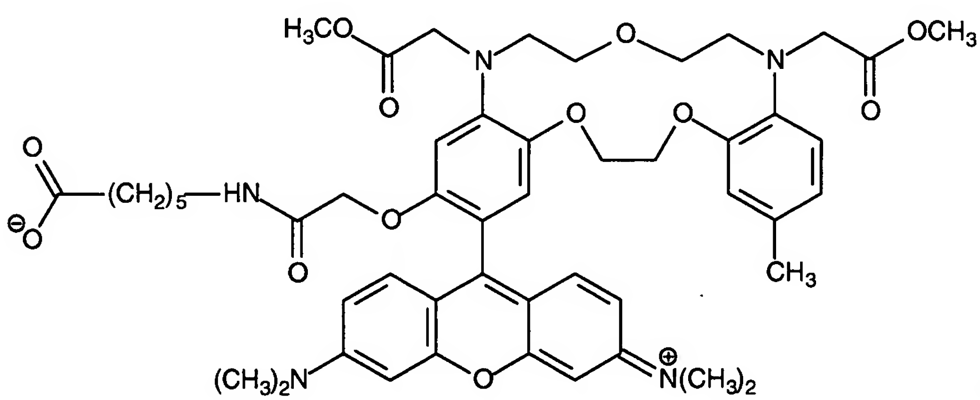
To a stirred solution of succinimidyl ester **155** prepared as in Example 36 from compound **153** (81 mg, 0.1 mmol) in DMF (2 mL) was added dry hexadecylamine (135 mg, 0.5 mmol). The mixture was stirred for 16 h, diluted with $CHCl_3$ (150 mL), washed with 1% AcOH the H_2O, dried over $MgSO_4$, and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using $CHCl_3$ as eluant to give Compound **156**, 57 mg (54%) as a red oil.



Compound **156**

Example 38. Preparation of 4-(N-5'-carboxypentylaminocarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (157).

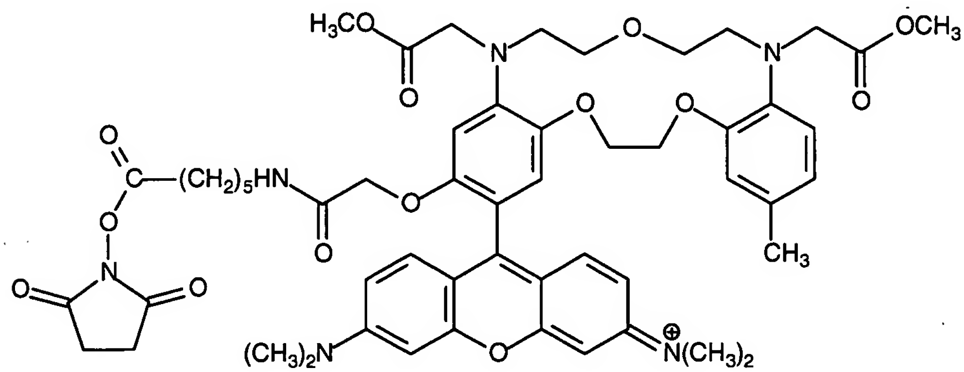
To a stirred solution of succinimidyl ester **155** prepared as in Example 36 from Compound **153** (81 mg, 0.1 mmol) in DMF (2 mL) was quickly introduced a solution of 5-aminopentanoic acid (40 mg, 0.3 mmol) and 1 M methanolic tetrabutylammonium hydroxide (1 mL, 1 mmol) in H_2O (5 mL). The mixture was stirred for 4 h, then diluted with H_2O (100 mL), acidified with AcOH to pH 4, extracted with $CHCl_3$, dried over $MgSO_4$ and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 10% MeOH and 2.5% AcOH in $CHCl_3$ as the eluant to give Compound **157**, 56 mg (61%) as a red solid.



Compound **157**

Example 39. Preparation of 4-(N-5'-Carboxypentylaminocarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, succinimidyl ester (158).

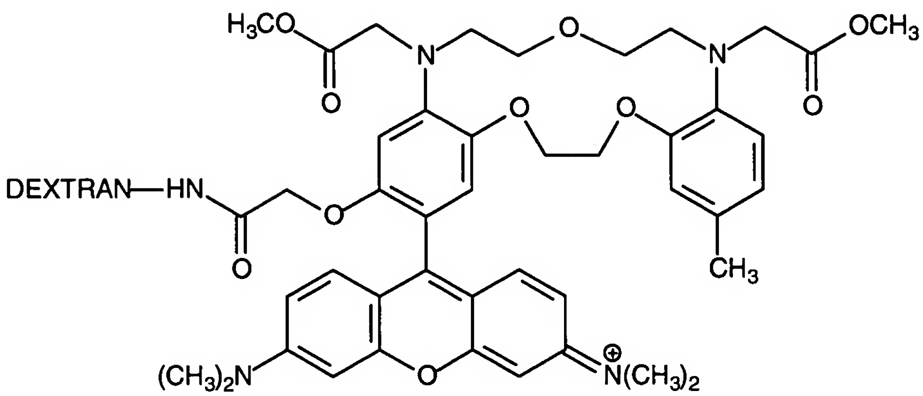
To a stirred solution of Compound **157** (37 mg, 0.04 mmol) and pyridine (0.08 mL, 1 mmol) in DMF (1 mL), was added dry N-trifluoroacetoxysuccinimide (36 mg, 0.16 mmol). After 4 h, more N-trifluoroacetoxysuccinimide (36 mg, 0.4 mmol) and pyridine (0.08 mL, 1 mmol) are added, and the mixture was stirred for an additional 16 h. The mixture was diluted with $CHCl_3$ (100 mL), washed with 1% AcOH then H_2O then evaporated. Hexanes (5 mL) were added to the residue. The precipitated solid was filtered, washed with hexanes and dried to give Compound **158**, 15 mg (37%) as a dark red solid.



Compound **158**

Example 40. Preparation of 4-carboxymethyloxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD, dextran conjugate (**160**).

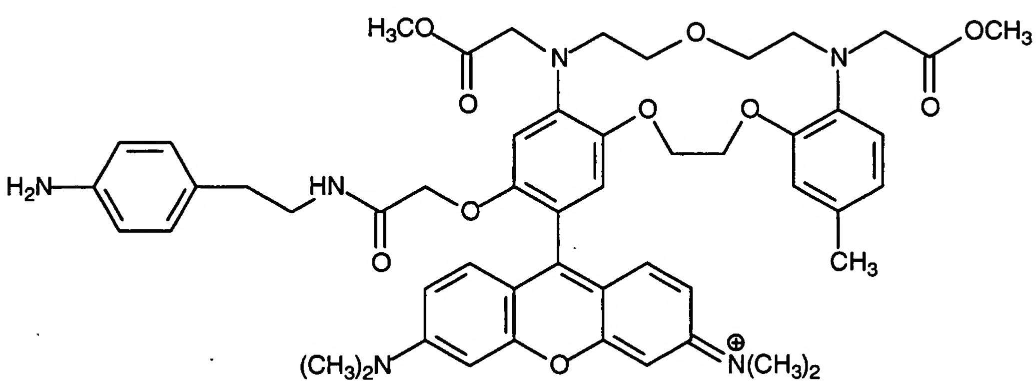
A solution of succinimidyl ester **155** (81 mg, 0.1 mmol) in DMF (2 mL) was added to 5 mL of aqueous aminodextran (100 mg, 0.037 eq.) and 1 M methanolic tetrabutylammonium hydroxide (1 mL, 1 mmol). The mixture was stirred for 16 h, poured into MeOH (400 mL), and the precipitated conjugate was filtered off and washed with MeOH. The crude product was dissolved in H_2O (3 mL), filtered through a membrane filter and loaded onto a SEPHADEX G-15 resin column pre-equilibrated with H_2O. The colored fraction was eluted in a void volume and lyophylized to give labeled dextran **160**, (41 mg) as a red solid.



Compound **160**

Example 41. Preparation of 4-(N-[(4''-aminophenyl)ethyl-2')carbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (**161**).

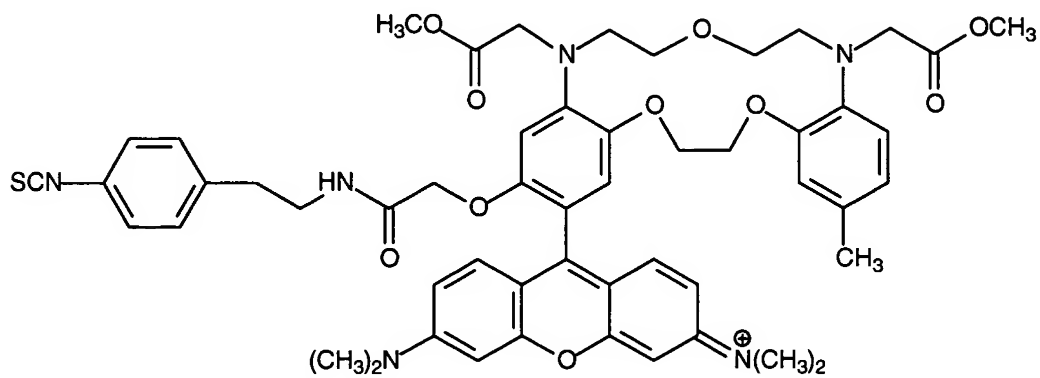
To a stirred solution of succinimidyl ester **155** (81 mg, 0.1 mmol) in DMF (2 mL) was added Et_3N (0.14 mL, 1 mmol) followed by 2-(4-aminophenyl)ethylamine (0.05 mL, 0.5 mmol). The mixture was stirred for 2 h, diluted with $CHCl_3$ (150 mL), washed with 1% AcOH then H_2O then evaporated. Ether (5 mL) was added to the residue, and the solid was filtered off, washed with ether and dried to give Compound **161** as a dark red solid, pure on TLC and HPLC.



Compound **161**

Example 42. Preparation of 4-(N-[(4''-isothiocyanatophenyl)ethyl-2')carbamoylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (**162**).

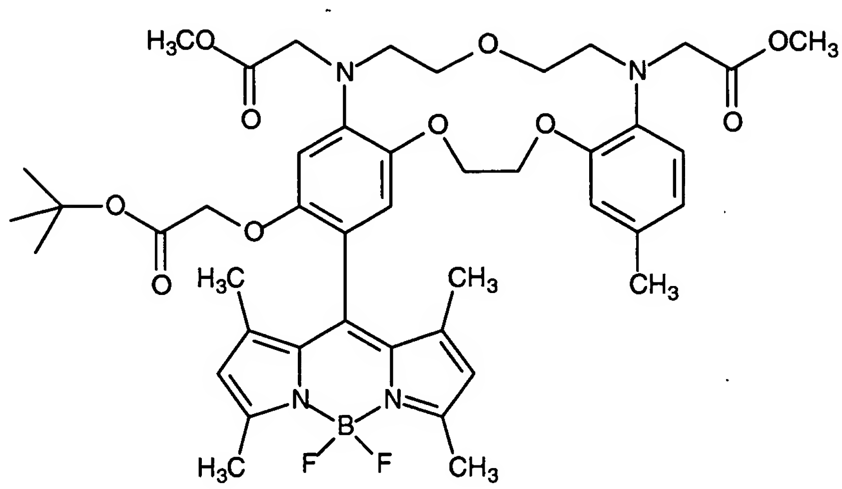
To a solution of Compound **161** (9 mg, 0.01 mmol) in AcOH (2 mL) was added a 1 M $CSCl_2$ solution in $CHCl_3$ (0.1 mL, 0.1 mmol). The mixture was stirred for 2 h then evaporated. Ether (5 mL) was added to the residue, and the precipitated solid was filtered off, washed with ether and dried to give Compound **162**, 9 mg (95%) as a dark red solid. Compound **162** reacts quickly with n-$BuNH_2$ in pre-column derivatization for HPLC analysis.



Compound **162**

Example 43. Preparation of 4-(*t*-butoxycarbonylmethyloxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-DDTCPD (**163**).

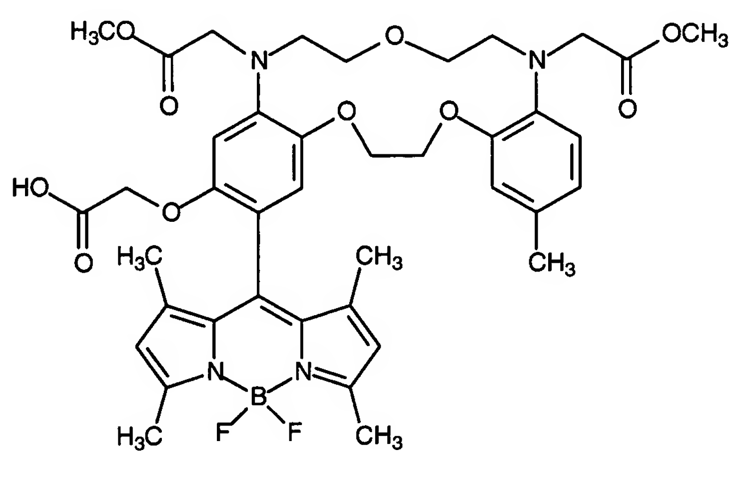
To a stirred solution of aldehyde **119** (630 mg, 1.0 mmol) in CH_2Cl_2 (40 mL), 2,4-dimethylpyrrole (0.25 mL, 2.4 mmol) was added. The solution was stirred for 5 min, then TFA (0.1 mL, 1.2 mmol) was introduced. The mixture was stirred for 16 h, and diluted with $CHCl_3$ (300 mL). The chloroform solution was washed with 2% tetrabutylammonium hydroxide then H_2O, evaporated, and subsequently co-evaporated with toluene. The residue was dissolved in toluene (40 mL), stirred for 2 h with chloranil (296 mg, 1.2 mmol), then DIPEA (1.74 mL, 10 mmol) was introduced, followed by BF_3 etherate (1.04 mL, 8 mmol). The mixture was evaporated and the residue was purified by chromatography on SiO_2 using a gradient of 0-1% MeOH in $CHCl_3$ to give Compound **163**, 280 mg (35%) as a brown solid.



Compound **163**

Example 44. Preparation of 4-(*t*-butoxycarbonylmethoxy)-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-DDTCPD (**164**).

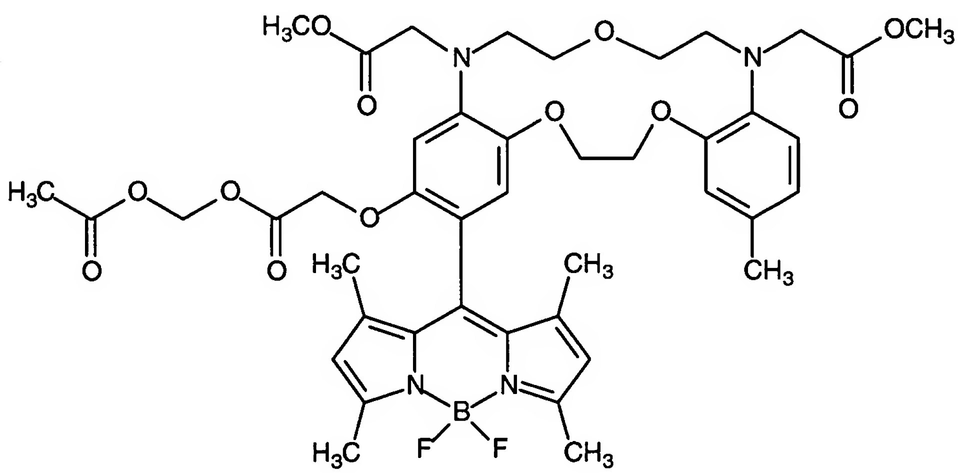
A mixture of Compound **163** (120 mg, 0.15 mmol) in $CHCl_3$ (6 mL) and TFA (0.4 mL) was stirred for 16 h, then evaporated and co-evaporated with $CHCl_3$. The residue was purified by preparative TLC on two SiO_2 plates using 10% MeOH in $CHCl_3$ as eluant to give Compound **164**, 82 mg (77%) as a brown solid.



Compound **164**

Example 45. Preparation of 4-acetoxymethoxycarbonylmethyloxy-1,18-bis(methoxycarbonylmethyl)-14-methyl-5-[4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacen-8-yl]-DDTCPD (**165**).

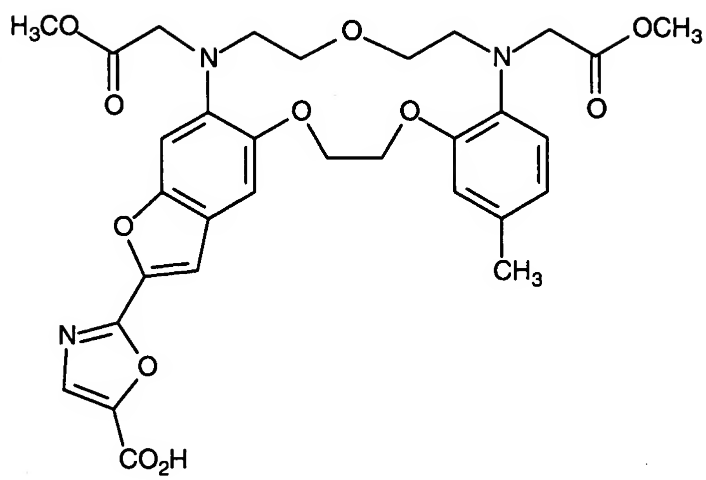
A mixture of Compound **164** (80 mg, 0.11 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.17 mL, 1.0 mmol) in DMF (2 mL) was stirred for 2 h, poured into H_2O (150 mL), and extracted with $CHCl_3$. The extract was dried over $MgSO_4$ then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 3% MeOH and 0.5% AcOH in $CHCl_3$ to give Compound **165**, 46 mg (52%) as a brown solid.



Compound **165**

Example 46. Preparation of Compound **167**.

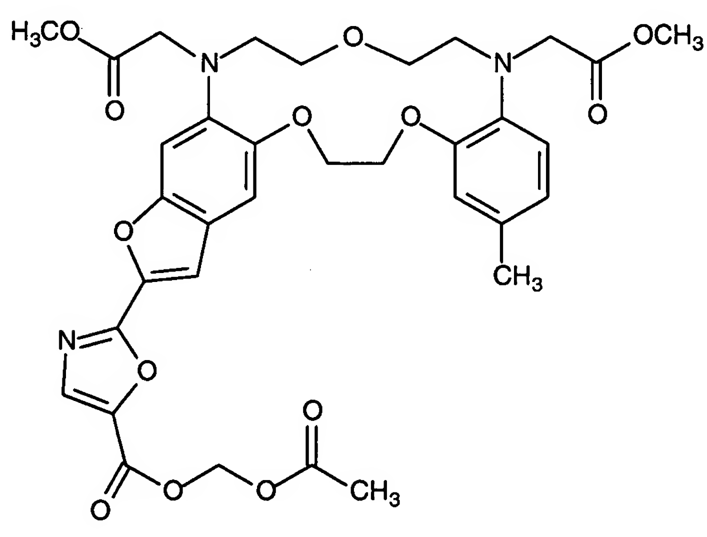
A mixture of aldehyde **118** (800 mg, 1.55 mmol), 5-carboxy-2-chloromethyloxazole (300 mg, 1.86 mmol), K_2CO_3 (1.07 g, 7.75 mmol), and NaI (75 mg, 0.5 mmol; catalyst) in DMF (20 mL) was stirred at 135 °C for 4 h, then poured into H_2O. The mixture was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$, dried over $MgSO_4$ then evaporated. The residue was purified by chromatography on SiO_2 using a gradient of 5–20% MeOH and 1-2% AcOH in $CHCl_3$ as eluant to give Compound **167**, 255 mg (26%) as a yellowish solid.



Compound **167**

Example 47. Preparation of Compound **168**.

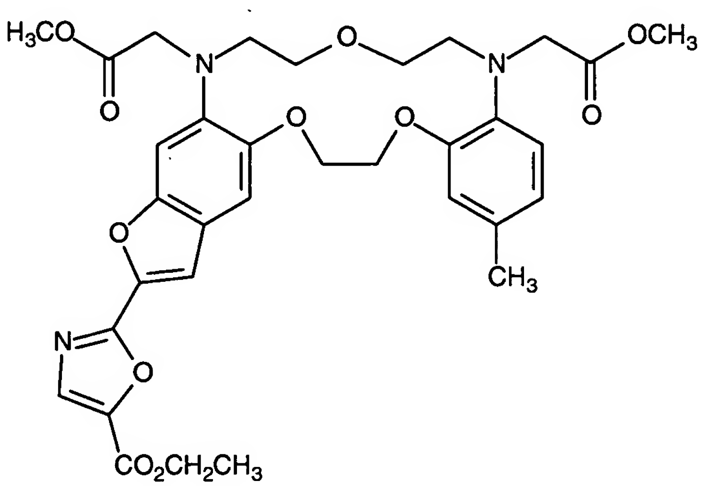
A mixture of Compound **167** (62 mg, 0.1 mmol), bromomethyl acetate (0.05 mL, 0.5 mmol), and DIPEA (0.17 mL, 1.0 mmol) in DMF (1 mL) was stirred for 2 h, poured into 1% AcOH (100 mL), and extracted with $CHCl_3$. Then 3 M NaOAc (100 mL) was added to the aqueous phase and the mixture was extracted with more $CHCl_3$. The combined extracts were dried over $MgSO_4$ then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give Compound **168**, 29 mg (42%) as an orange solid.



Compound **168**

Example 48. Preparation of Compound **170**.

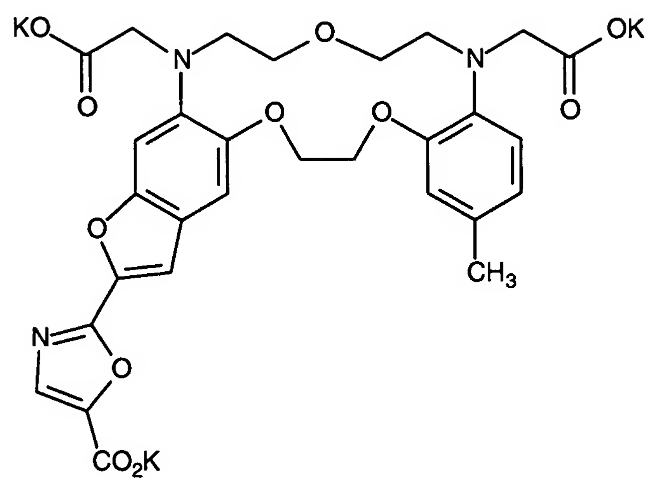
A mixture of aldehyde **118** (1.40 g, 2.71 mmol), 2-chloromethyl-5-ethoxycarbonyloxazole (0.564 g, 1.86 mmol), K_2CO_3 (1.87 g, 13.60 mmol), and NaI (0.150 g, 1.00 mmol; catalyst) in DMF (30 mL) was stirred at 135 °C for 4 h, then poured into H_2O. The mixture was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$ and evaporated. The residue was purified by chromatography on SiO_2 using 1% MeOH in $CHCl_3$ as eluant to give Compound **170**, 1.260 g (71%) as a yellowish solid.



Compound **170**

Example 49. Preparation of Compound **171**.

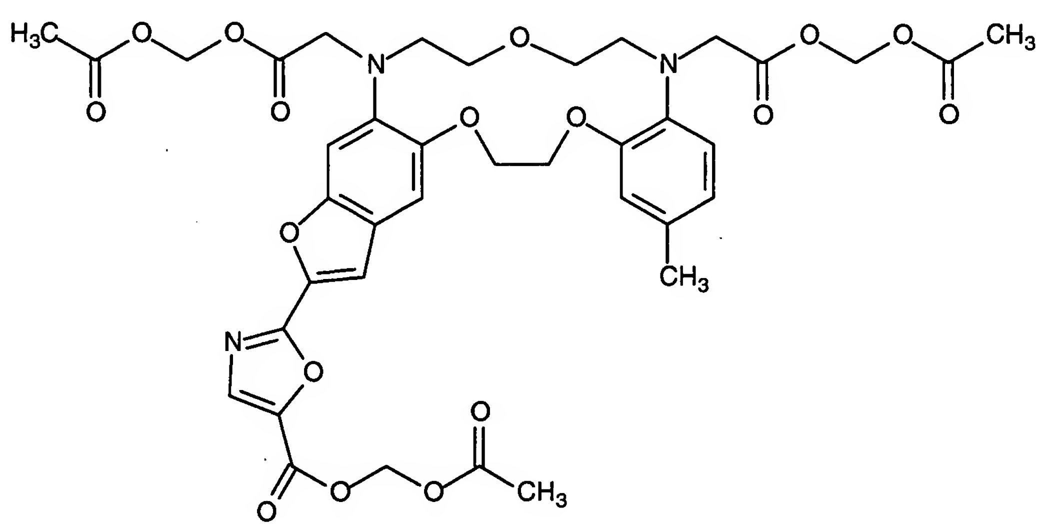
A mixture of Compound **170** (376 mg, 0.5 mmol) and 1 M KOH (3 mL, 3.0 mmol) in MeOH (5 mL) was stirred for 5 h, then evaporated to 1/5 volume, and acidified with 1 M HCl to pH 2.8. The precipitated solid was filtered, washed with H_2O and dried on a filter. This crude product was suspended in H_2O (2 mL), then made basic with 0.1 M KOH to pH 9.5. The solution was loaded onto a SEPHADEX LH-20 resin column and chromatographed using H_2O as eluant to give Compound **171**, 256 mg (72%) as a yellow-greenish solid.



Compound **171**

Example 50. Preparation of Compound **172.**

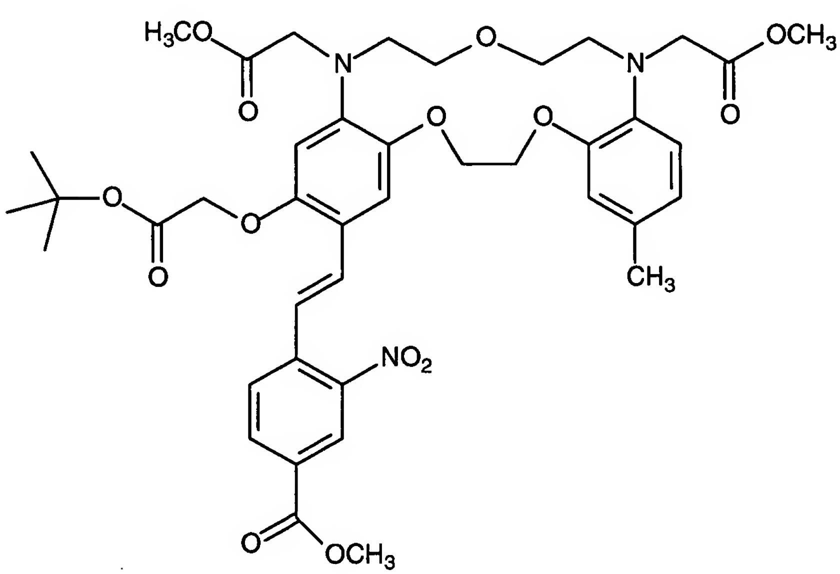
A mixture of Compound **171** (71 mg, 0.1 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.35 mL, 2.0 mmol) in DMF (2 mL) was stirred for 4 h, diluted with $CHCl_3$, then washed with 1% AcOH then H_2O and evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH and 0.5% AcOH in $CHCl_3$ to give compound **172**, 63 mg (77%) as an orange solid.



Compound **172**

Example 51. Preparation of trans 1-[4'-*t*-butoxycarbonylmethyloxy-1',18'-bis-(methoxycarbonylmethyl)-14'-methyl-DDTCPD-5-yl]-2-[4''-methoxycarbonyl-2''-nitrophenyl] ethylene (**173**).

A mixture of aldehyde **119** (315 mg, 0.5 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (375 mg, 0.7 mmol), and K_2CO_3 (345 mg, 2.5 mmol) in DMF (3 mL) was stirred for 6 h at 95 °C then cooled to room temperature and poured into H_2O. The solution was acidified to pH 5 with 1 M HCl and extracted with $CHCl_3$. The extract was dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography on SiO_2 using $CHCl_3$, then 0.5% MeOH in $CHCl_3$ to give Compound **173**, 339 mg (84%) as an orange solid.

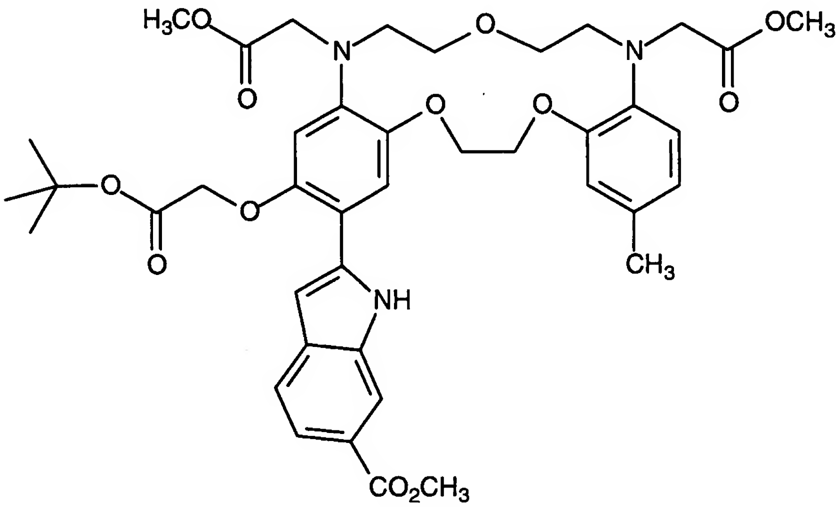


Compound **173**

Example 52. Preparation of 4-*t*-butoxycarbonylmethyloxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (**174**).

A mixture of the ethylene derivative **173** (338 mg, 0.42 mmol) and triethylphosphite (8 mL) was heated at 130 °C for 7 h, then evaporated and subsequently co-evaporated with DMF. The residue was first purified by chromatography on SiO_2 using $CHCl_3$ followed by 1%

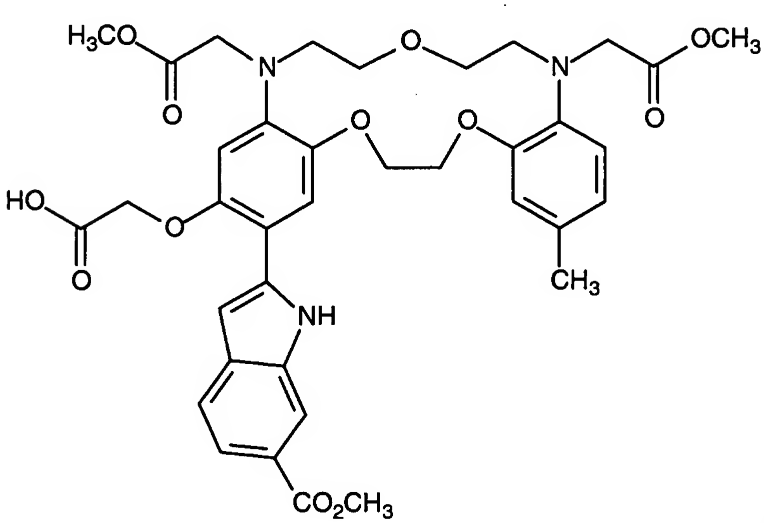
MeOH in $CHCl_3$, then by preparative TLC on two SiO_2 plates using 50% EtOAc in hexanes as eluant to give Compound **174**, 112 mg (34%) as a slightly yellowish solid.



Compound **174**

Example 53. Preparation of 4-carboxymethyloxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (**175**).

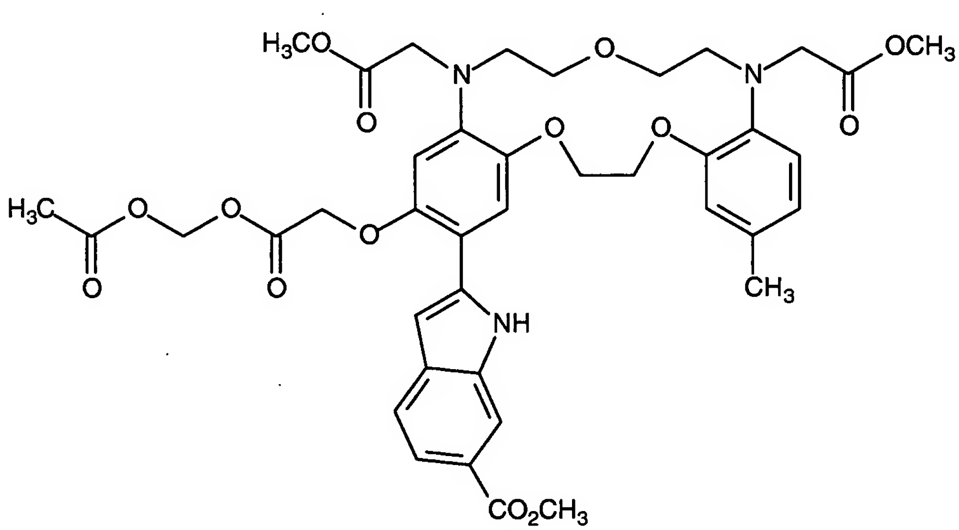
A mixture of Compound **174** (104 mg, 0.13 mmol) in CH_2Cl_2 (2 mL) and TFA (2 mL) was stirred for 3 h, then evaporated and co-evaporated with $CHCl_3$. The residue was purified by preparative TLC on two SiO_2 plates using 7% MeOH and 2% AcOH in $CHCl_3$ to give Compound **175**, 55 mg (57%) as a yellowish solid.



Compound **175**

Example 54. Preparation of 4-acetoxymethylcarbonylmethyloxy 1,18-bis-(methoxycarbonylmethyl)-5-(6'- methoxycarbonylindolyl-2')-14-methyl-DDTCPD (176).

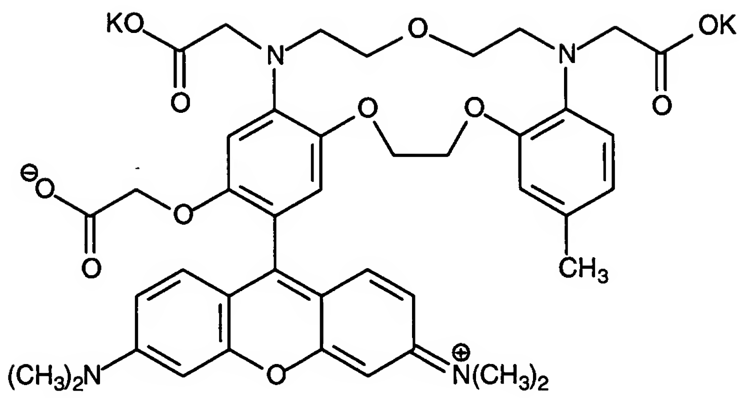
A mixture of Compound **175** (22 mg, 0.03 mmol), bromomethyl acetate (0.1 mL, 1.0 mmol), and DIPEA (0.26 mL, 1.5 mmol) in DMF (2 mL) was stirred for 16 h, then diluted with $CHCl_3$ (100 mL), washed with 1% AcOH then H_2O then evaporated. The residue was purified by preparative TLC on two SiO_2 plates using 5% MeOH in $CHCl_3$ to give compound **176**, 16 mg (67%) as an orange solid.



Compound **176**

Example 55. Preparation of 4-carboxymethyloxy-1,18-bis(carboxymethyl)-14-methyl-5-[TMR]-DDTCPD (177).

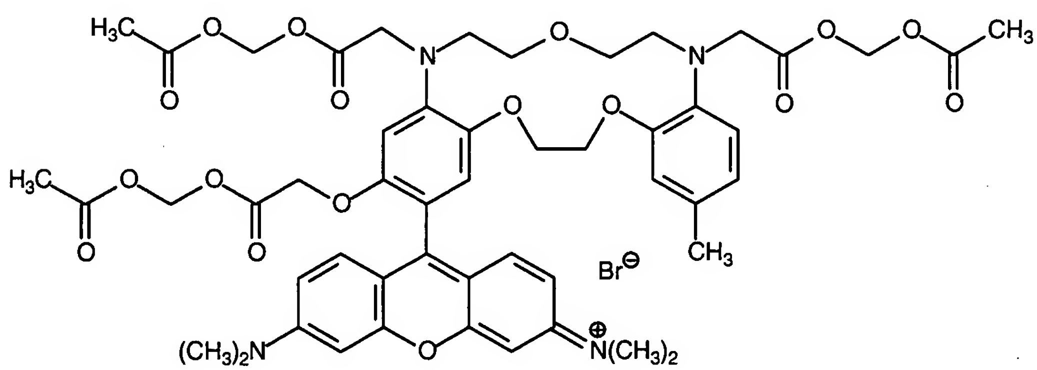
A mixture of Compound **152** (87 mg, 0.1 mmol) and 1 M KOH (2 mL, 2 mmol) in MeOH (5 mL) was stirred for 16 h, and evaporated. The residue was purified on SEPHADEX LH-20 resin using H_2O as the eluant. The fractions containing product were collected and lyophilized to give Compound **177**, 12 mg (14%) as a crimson solid.



Compound **177**

Example 56. Preparation of 4-acetoxymethoxycarbonylmethyloxy-1,18-bis(acetoxymethoxycarbonylmethyl)-14-methyl-5-[TMR]-DDTCPD (178).

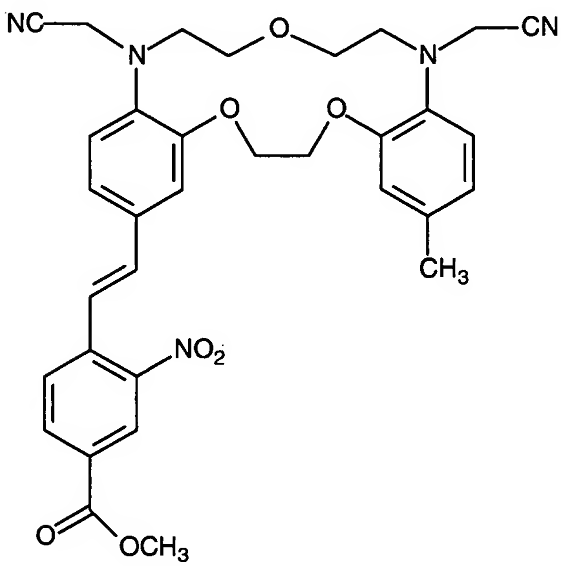
A mixture of Compound **177** (8 mg, 0.01 mmol), bromomethyl acetate (0.025 mL, 0.25 mmol), and DIPEA (0.08 mL, 0.5 mmol) in DMF (1 mL) was stirred for 16 h, and diluted with $CHCl_3$ (100 mL). The solution was washed with 1% AcOH then H_2O then evaporated. Ether (5 mL) was added to the residue. The precipitated solid was filtered off and washed with ether to give Compound **178**, 5 mg (50%) as a dark red solid. Compound **178** was pure on TLC and HPLC.



Compound **178**

Example 57. Preparation of trans 1-[1',18'-bis-(cyanomethyl)-14'-methyl-DDTCPD-5-yl]-2-[4''-methoxycarbonyl-2''-nitrophenyl] ethylene (179).

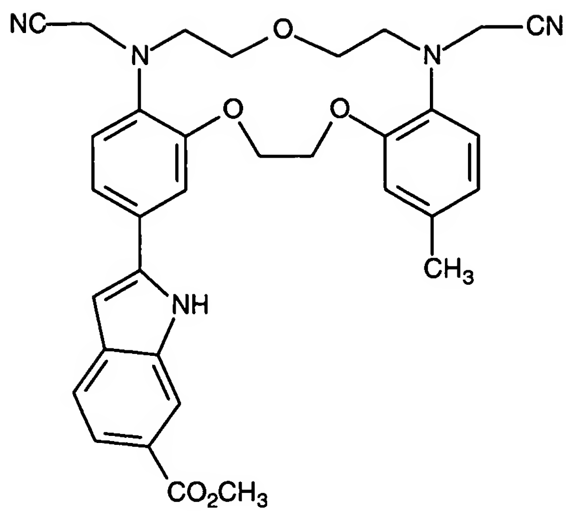
A mixture of aldehyde **121** (217 mg, 0.5 mmol), the Wittig base (4-methoxycarbonyl-2-nitrobenzyl)triphenylphosphonium bromide (536 mg, 0.7 mmol), and K_2CO_3 (345 mg, 2.5 mmol) in DMF (3 mL) was stirred for 16 h at 95 °C, then cooled to room temperature and poured into H_2O. The solution was acidified with 1 M HCl to pH 3, extracted with $CHCl_3$, dried over $MgSO_4$ and evaporated. The crude product was purified by chromatography on SiO_2 using $CHCl_3$, as eluant to give Compound **179**, 171 mg (56%) as an orange solid.



Compound **179**

Example 58. Preparation of 1,18-bis(cyanomethyl)-5-(6'-methoxycarbonylindolyl-2')-14-methyl-DDTCPD (**180**).

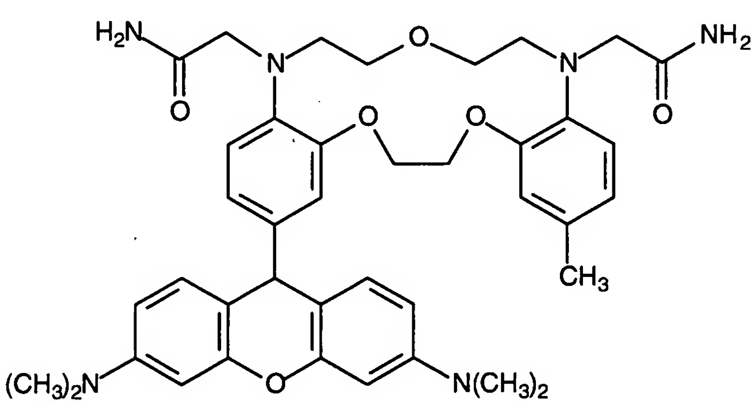
A mixture of the ethylene derivative **179** (170 mg, 0.28 mmol) and triethylphosphite (4 mL) was heated at 120 °C for 16 h, then evaporated and subsequently co-evaporated with DMF. The residue was purified by preparative TLC on two SiO_2 plates using 3% MeOH in $CHCl_3$ as eluant, then on two SiO_2 plates using 40% EtOAc in hexanes as eluant to give Compound **180**, 26 mg (16%) as a yellowish solid.



Compound **180**

Example 59. Preparation of 1,18-bis(carbamoylmethyl)-14-methyl-5-[dihydrotetramethylrhodamine]-DDTCPD (181).

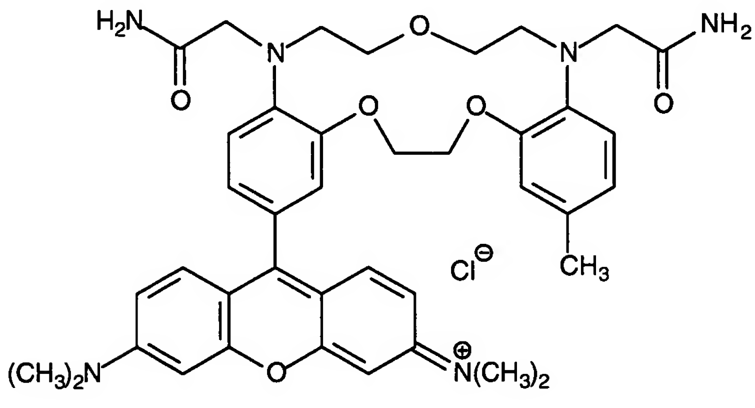
A mixture of aldehyde **123** (100 mg, 0.21 mmol), 3-dimethylaminophenol (55 mg, 0.40 mmol), and p-toluenesulphonic acid (5 mg, catalyst) in propionic acid (2 mL) was stirred overnight at 60 °C, then cooled to room temperature and poured into 3 M NaOAc (40 mL). Precipitated solid was filtered, washed with H_2O and dried to give Compound **181,** 140 mg (93%) as a rose-colored solid. Compound **181** was unstable to oxidation and was used in next step without additional purification.



Compound **181**

Example 60. Preparation of 1,18-bis(carbamoylmethyl)-14-methyl-5-[tetramethylrhodamine]-DDTCPD (**182**).

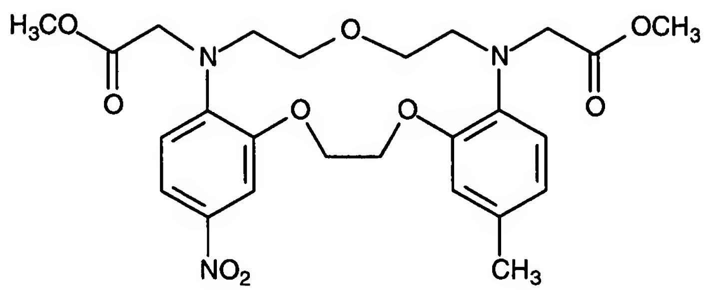
A mixture of Compound **181** (138 mg, 0.20 mmol) and chloranil (84 mg, 0.34 mmol) in MeOH (5 mL) and $CHCl_3$ (5 mL) was stirred for 4 h, and evaporated. The residue was purified by chromatography on SiO_2 using a gradient of 7-9% MeOH and 0.5-1% AcOH in $CHCl_3$ as eluant to give Compound **182,** 118 mg (86%) as a dark red solid.



Compound **182**

Example 61. Preparation of Compound **200**.

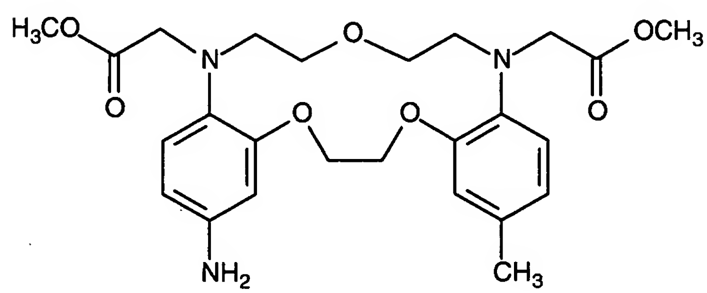
To a 0.5 M solution of arene **108** in sulfuric acid is added one equivalent of potassium nitrate. The resulting solution is stirred until TLC analysis of reaction aliquots, treated with water and ether, shows full conversion to nitroarene **200**. The reaction mixture is poured into excess aqueous sodium acetate, followed by extraction with ether. The extract is washed with aqueous sodium bicarbonate and brine, dried over magnesium sulfate, then concentrated to a reddish-brown oil, which is purified by trituration with ether-hexanes to give Compound **200** as a yellow powder.



Compound **200**

Example 62. Preparation of Compound **201.**

A 0.5 M solution of Compound **200** in methanol is treated with 10% palladium on charcaol, at 10 wt% of Compound **200**. The resulting mixture is shaken under 40 psi hydrogen until TLC analysis shows full conversion to aniline **201**. The reaction mixture is filtered through diatomaceous earth, followed by concentration in vacuo. The residue is triturated with ether-hexanes to give pure Compound **201** as an off-white powder.

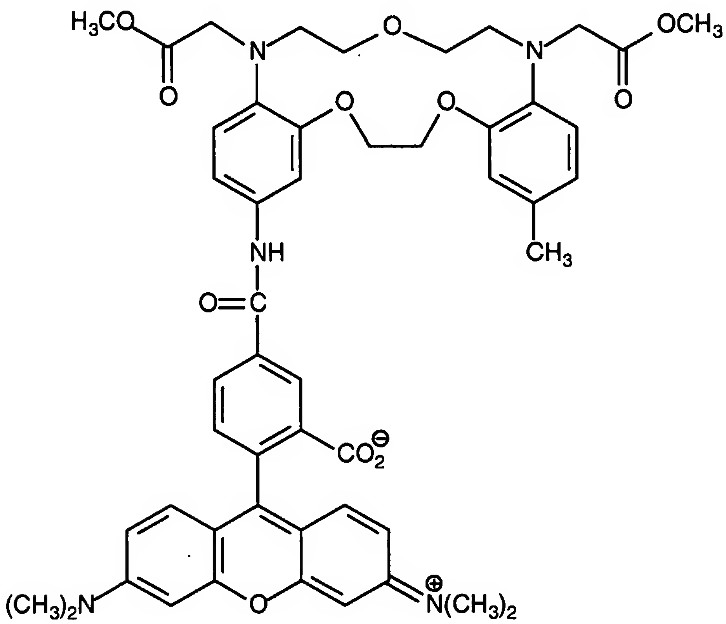


Compound **201**

Example 63. Preparation of Compound **203.**

A mixed anhydride of 6-carboxytetramethylrhodamine is prepared according to the procedure given in U.S. Patent No. 5,453,517 to Kuhn et al. To a solution of the mixed anhydride and one equivalent of diisopropylethylamine in anhydrous THF under nitrogen is added slowly a 0.3 M solution of Compound **201**. The resulting mixture is stirred until TLC analysis indicates consumption of Compound **201**. The reaction mixture is concentrated in vacuo, and the residue partitioned between chloroform and water. The chloroform layer is washed with brine and dried over magnesium sulfate, then concentrated in vacuo. The residue is purified

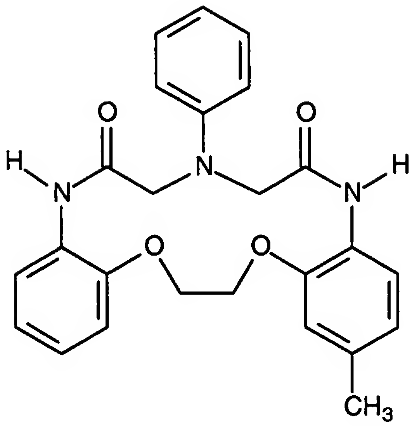
by flash chromatography on silica gel using increasing amounts of methanol in chloroform as eluant to give pure Compound **203** as a red powder.



Compound **203**

Example 64. Preparation of a tris-aza crown ether (**205**).

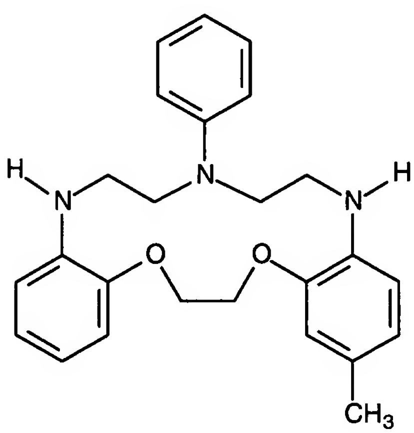
To a dilute solution (0.1 M) of diamine **105** and two equivalents of DIPEA in anhydrous THF is slowly added at room temperature a dilute solution (0.1 M) of N,N-bis(chlorocarbonylmethyl)-aniline. After stirring overnight, the volatiles are removed by evaporation, and the residue is partitioned between 5% hydrochloric acid and ethyl acetate. The organic layer is washed with brine, dried, and concentrated. The resulting residue is purified by column chromatography on silica gel using increasing amounts of methanol in chloroform to give pure Compound **205**.



Compound **205**

Example 65. Preparation of Compound **206.**

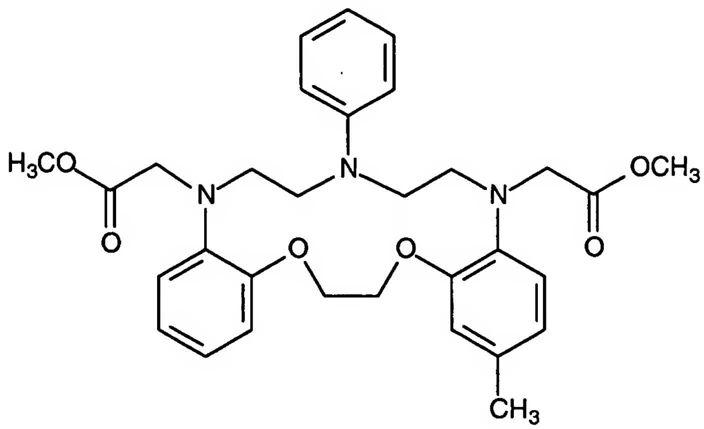
The bisamide **205** is reduced to bisaniline **206** as described for Compound **107**.



Compound **206**

Example 66. Preparation of Compound **207.**

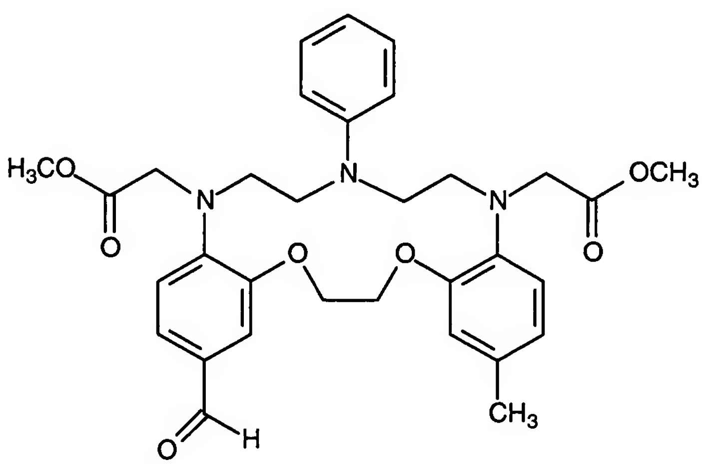
The bisaniline **206** is alkylated to give Compound **207**, as described for Compound **108**.



Compound **207**

Example 67. Preparation of Compound 208.

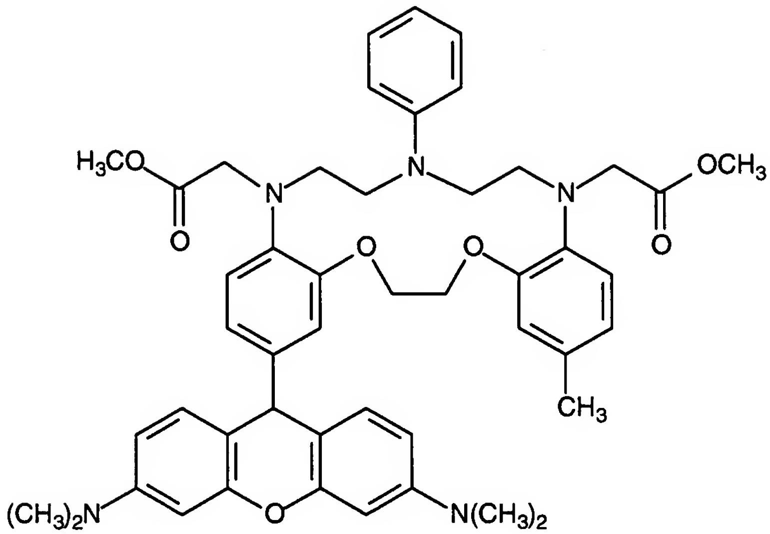
The bisester **207** is formylated to give the aldehyde Compound **208**, as described for Compound **109**.



Compound **208**

Example 68. Preparation of Compound 209.

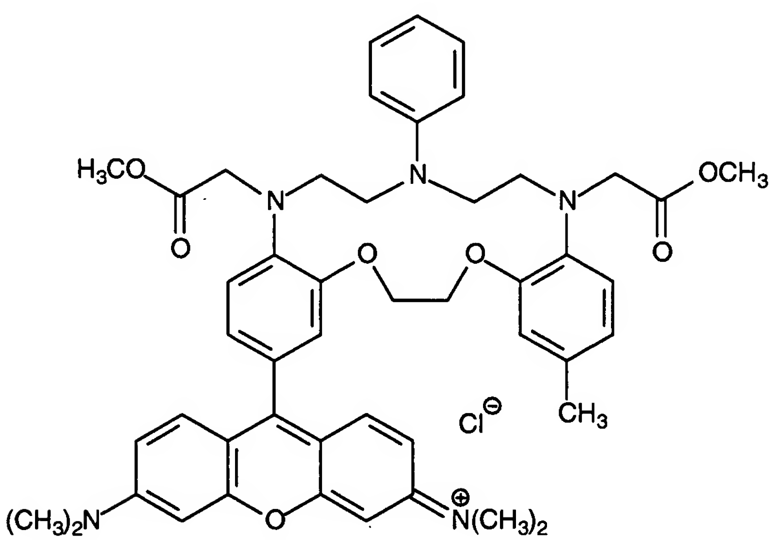
The aldehyde **208** is condensed with two equivalent of 3-(dimethylamino)phenol as described for Compound **137** to give Compound **209**.



Compound **209**

<u>Example 69. Preparation of Compound **210**.</u>

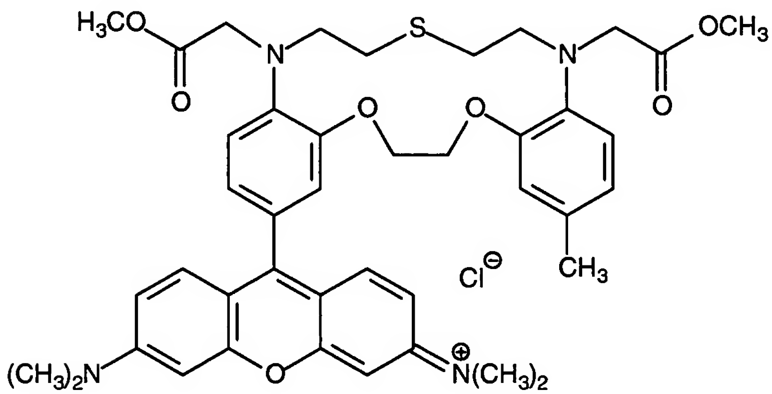
Compound **209** is oxidized with chloranil to give the chloride salt **210** as a red powder, as described for Compound **138**.



Compound **210**

<u>Example 70. Preparation of a thia-substituted crown ether (**211**).</u>

A bis-aza crown ether that incorporates a sulfur atom in the crown is prepared using the procedures of Examples 64-69, except that in place of N,N-bis(chlorocarbonylmethyl)-aniline, the bis-acid chloride of thiodiglycolic acid is used ($Cl(C=O)CH_2$-S-$CH_2(C=O)Cl$). After preparation of the crown ether itself, condensation with 3-(N,N-dimethylamino)phenol, and oxidation, the chloride salt of the thia-crown ether, Compound **211**, is isolated as a red powder.



Compound **211**

Example 71. Determination of sodium indicator fluorescence response.

The fluorescence response of a selected compound of the invention as a function of sodium ion concentration was determined by dissolving a sample of the pure compound in 3 mL of each of two solutions: solution 1 ("high Na^+") consists of 200 mM NaCl and 10 mM MOPS buffer at pH 7.05; solution 2 ("zero Na^+") consists of 10 mM MOPS buffer at pH 7.05 in deionized water. A series of curves are generated by cross dilution between the two solutions to arrive at intermediate concentrations of Na^+. The emission of a selected indicator in solution 2 was scanned and then was repeated to cover the entire range from zero to 200 mM Na^+. For example, the fluorescence emission of the chosen indicator was scanned while the sample was excited at that indicator's absorption maximum wavelength, and then 1/100 of the sample was removed and replaced with 1/100 of solution 1 to arrive at a Na^+ concentration of 2 mM. This dilution was repeated to cover the entire range from zero to 1 M Na^+ and the resulting emission intensities were plotted versus the ion concentrations. A least-squares fit was used to arrive at the concentration where the selected indicator was maximally sensitive to changes in Na^+

concentration. This corresponded to the dissociation constant of that indicator for Na^+ and was expressed as a concentration.

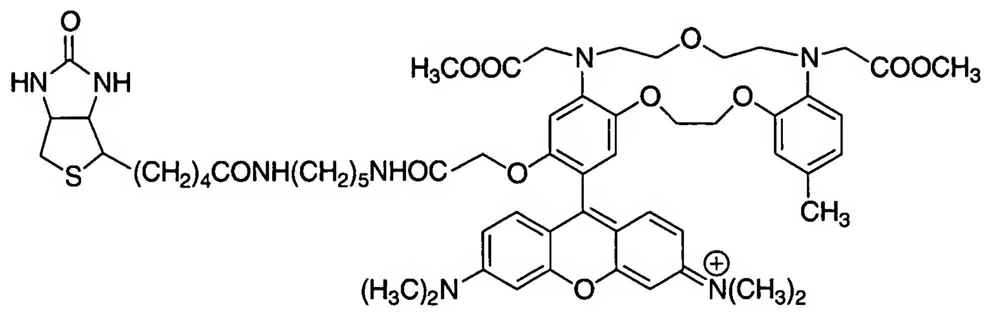
For visible wavelength probes such as Compound **132** (Example 17) and Compound **138** (Example 22) the indicator's fluorescence emission was typically scanned from 450-650 nm while the sample was excited at the absorption maximum wavelength. For UV-excitable ratiometric probes such as Compound **167** (Example 46), the excitation wavelengths of the dye in solution 2 were scanned from 260 to 450 nm while monitoring constant fluorescence emission at 510 nm (as in Figure 1).

The binding affinity of a selected indicator for sodium ions, in the presence of potassium ions, is determined using the process above, except in the presence of 100 mM K^+ concentrations.

Example 72. Calibration of sodium indicator fluorescence response in cells.

Jurkat cells were loaded with a 5 μM solution of either Compound **135** (Example 20) or commercially available SODIUM GREEN tetraacetate indicator (Molecular Probes, Inc., Eugene, OR) for 30 minutes at 37 °C. The use of PLURONIC dispersing agent helped dissolve the selected indicator. Intracellular sodium concentrations were then established by varying the sodium concentration of the extracellular buffer in the presence of 2 μM gramicidin (a sodium-pore forming antibiotic). The extracellular buffer was set at 0 mM, 10 mM, 20 mM, 50 mM, 100 mM, and 145 mM, respectively. Intracellular fluorescence response of the selected indicators was measured using a FACSCAN flow cytometer and associated software, with fluorescence excitation at 488 nm. Compound **135** exhibits substantially brighter intracellular fluorescence intensity than the SODIUM GREEN indicator at comparable Na^+ concentrations, as shown in Figure 3. Similarly, as intracellular Na^+ concentration was increased, Compound **135** exhibits a consistent increase in fluorescence intensity.

Example 73. Preparation of a biotinylated indicator, Compound 212.



Compound **212**

To a stirred solution of succinimidyl ester 155 prepared as in Example 36 from compound 153 (81 mg, 0.1 mmol) in DMF (2 mL) was added Et_3N (0.27 mL, 2 mmol) followed by biotin-cadaverin trifluoracetate (66 mg, 02 mmol). The mixture was stirred for 16 h, diluted with $CHCl_3$ (100 mL), washed 1% AcOH (3 x 50 mL), H_2O (100 mL) dried over $MgSO_4$ and evaporated. The residue was purified by preparative TLC on four C18 reverse-phase plates using 50% aqueous 2-PrOH with 0.2% TFA as eluant to give Compound 212, 25 mg (22%) as an orange solid.

Example 74. **Avidin-labeling with a biotinylated fluorescent crown ether.**

A 5 mg/mL solution of streptavidin in phosphate-buffered saline (PBS, pH 7.0) is treated with a 1 mg/mL solution of biotinylated indicator (e.g., Compound 212, Example 73) in 2% DMSO/PBS at a molar ratio such that two equivalents of biotinylated indicator are present for every streptavidin molecule. The resulting solution is incubated at 37°C for 4 hours, then centrifuged. The supernatant is applied to a Sephadex G-25 gel filtration column (2 mL bed volume/mg protein) and eluted with PBS. The streptavidin-indicator complex elutes first. Fractions are analyzed by TLC to ensure that no free indicator is present in the complex. Pure product fractions are pooled and lyophilized. The complex is useful as a bridging method to apply the indicator to any biotinylated surface or substance.

All publications, patents and patent applications referred to within this document are incorporated by reference to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference.

The reagents employed in the examples are commercially available or can be prepared using commercially available instrumentation, methods, or reagents known in the art. The foregoing examples illustrate various aspects of the invention and practice of the methods of the invention. The examples are not intended to provide an exhaustive description of the many different embodiments of the invention. Thus, although the forgoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, those of ordinary skill in the art will realize readily that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.